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(54) Title: TEICHOIC ACID ENZYMES AND ASSAYS

(57) Abstract

This invention discloses a novel substrate and assay for the TAP enzyme. In addition novel DNA, proteins and peptides from genes and proteins associated with bacterial teichoic acid biosynthetic pathways, specifically the *rodC* gene and proteins and variations thereof are disclosed.

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TEICHOIC ACID ENZYMES AND ASSAYS

Field of the Invention

This invention relates to the field of cell biology, more specifically the teichoic acid pathway. Genes and proteins related to this pathway include: Teichoic Acid Polymerase (or TAP), and CDP-Glycerol:Poly(glycerophosphate) Glycerophosphotransferase.

Information Disclosure

A.L.Honeyman, G.C. Stewart, "Identification of the protein encoded by *rodC*, a cell division gene from *Bacillus subtilis*" *Mol. Microbiol.* (1988) 2:735-741.

A.L.Honeyman, G.C. Stewart, "The nucleotide sequence of the *rodC* operon of *Bacillus subtilis*. *Mol. Microbiol.* (1989) 3:1257-1268.

C. Mauel, M. Young, P. Margot, D. Karamata "The essential nature of teichoic acids in *Bacillus subtilis* as revealed by insertional mutagenesis" *Mol. Gen. Genet.* (1991) 215:388-394.

C. Mauel, M.Young, D. Karamata, "Genes concerned with synthesis of poly(glycerol phosphate), the essential teichoic acid in *Bacillus subtilis* strain 168, are organized in two divergent transcription units" *J. Gen. Microbiol.* (1991) 137:929-941.

Y.S. Park, T.D. Sweitzer, J.E. Kison, C. Kent. "Expression, purification, and characterization of CTP:Glycerol-3-phosphate cytidyltransferase from *Bacillus subtilis*." *J. Biol. Chem.* (1993) 268:16648-16654.

Background of the Invention

The spread of antibiotic resistance in gram positive pathogenic bacteria is a serious problem which is only beginning to be registered in the clinic. The incidence of drug resistance is increasing - especially in *Staphylococcus aureus*, *Streptococcus pneumonia*, and the enterococci. Methicillin resistant *S. aureus* (MRSA), penicillin resistant *S. pneumoniae*, and vancomycin resistant enterococci, pose a serious threat to compromised patients. Vancomycin is the only antibiotic effective against MRSA. See, C.T. Walsh, "Vancomycin resistance: decoding the molecular logic" *Science* (1993) 261:308-309; I.R. Friedland, "Therapy of penicillin- and cephalosporin-resistant pneumococcal infections" *Trends Clinic Pract.* (1993) 25:451-455 and S. Dutka-Malen, and P. Courvalin, "Update on glycopeptide resistance in enterococci" *Antimicrob News* (1990) 7:81-88.

The cell wall teichoic acid pathway is found in the majority of gram positive bacteria, and studies with *Bacillus subtilis* have revealed that it is essential to cell viability. See, C. Mauel, M. Young, P. Margot, D. Karamata, "The essential nature of teichoic acids in *Bacillus subtilis* as revealed by insertional mutagenesis" *Mol Gen Genet* (1991) 215:388-394. The essential nature of cell wall teichoic acid may be due to the covalent attachment that it forms with peptidoglycan.

Cell wall teichoic acid, like peptidoglycan, is synthesized at the outer surface of the cell membrane using a nucleotide precursor (CDPglycerol) as the building block. Teichoic acid is a polymer of polyglycerolphosphate that is covalently attached to the peptidoglycan of gram positive bacteria. The enzyme CDP-Glycerol: Poly(glycerophosphate) glycerophosphotransferase catalyzes the polymerization of glycerolphosphate monomers from CDP-glycerol into a chain of polyglycerolphosphate linked via 1,3-phosphodiester bonds. Lipoteichoic acid is a related polymer of polyglycerolphosphate which is anchored to the cell membrane but is not attached to peptidoglycan.

There is an obvious clinical need for new antimicrobial agents which inhibit novel targets. In order to screen for unique inhibitors, essential metabolic pathways of gram positive pathogens, such as the cell wall teichoic acid pathway must be identified and their respective enzymes studied, cloned and made into useful assays and screens in order to identify novel antimicrobial agents.

Summary of the Invention

This invention discloses a method of measuring and assaying the activity of the TAP enzyme. This invention also demonstrates how a common commercially available material may be used as a substrate for an important biological reaction that has previously had no substrate available for evaluating this reaction. This invention teaches the researcher and clinician that lipoteichoic can be used as a substrate to elucidate the presence and even the activity of the TAP enzyme. An embodiment of this invention is the application of this teaching to create an assay that enables one to monitor the activity of the TAP enzyme.

This invention also discloses, for the first time, the sequence of an active TAP enzyme and the nucleic acid sequence of the DNA that codes for this sequence.

This invention includes: the entire DNA sequence shown in Sequence Chart 1 and Sequence Listing I.D. no. 1, and the DNA from residues 4 to 2274, first to last restriction site, and the DNA residues 24 to 2264. The coding DNA sequence shown in Sequence Chart 1, alternatively named, "the *rodC* gene." The DNA sequences corresponding to the sequence in Sequence Chart 1 where the residue at

position 1872 is tyrosine in place of cytosine.

A bacterial DNA sequence that is capable of hybridizing to the DNA sequence of Sequence Chart 1, under standard stringent conditions, to about 70 or more including, 75, 80, 85, 90, 95 or greater percent homology and having the ability to catalyze the reaction of CDP-glycerol plus H_2O into teichoic or lipoteichoic acid.

The DNA sequence from *Staphylococcus aureus* that codes for the protein or protein sequence fragment from *Staphylococcus aureus* having at least 70% homology to related fragments described by Sequence Charts 1 and 2, and that yield fragments of 7.0 kb, 5 kb, and 4.2 kb after EcoRI digest, or that yield fragments of 4.5, 3.3, 2.8 kb, after HindIII digest.

In addition to the DNA sequence, this invention describes various mutants, including: A collection of randomly mutated *rodC* genes. A selection of one or more randomly mutated *rodC* genes. A collection of bacteria having randomly mutated *rodC* genes. A selection of one or more bacteria having a random mutation selected from the collection of bacteria. The mutated bacteria selected from a mutant form of *B. subtilis* or *S. aureus*.

Various proteins and peptide fragments from the expressed DNA are also described. The entire protein sequence shown in Sequence Charts 1 and 2, Sequence I.D. NO. 2, the protein sequence from residues 1- 746, and the protein sequence shown in Sequence Charts 1 and 2 where valine is the amino acid at position 616 in place of alanine. Also described are the protein sequence fragment from *Staphylococcus aureus* having at least 70% homology to related fragments described by Sequence Charts 1 and 2, that yield fragments of 7.0 kb, 5 kb, and 4.2 kb after EcoRI digest; and the protein sequence fragment from *Staphylococcus aureus* having at least 70 % homology to related fragments described by Sequence Charts 1 and 2, that yield fragments of 4.5, 3.3, 2.8 kb, after HindIII digest. The protein disclosed in the Southern Blot shown in Figure 2 is described as well..

In addition to the DNA and proteins disclosed herein, this invention comprises various intermediates, intermediate vectors, plasmids and transformed or mutated cell lines. This invention comprises the DNA of the sequence disclosed in Sequence Chart 1 incorporated into a vector selected from a cloning vector, a shuttle vector or an expression vector, any of these vectors may be plasmid vectors. The cloning vector or plasmid can be selected from any widely available or commercially available plasmids. The plasmid can be any suitable pUC type or pBR type of plasmid, such as pUC18, or pUC19, or any other suitable plasmid such as pBR322. The vector may be a typical shuttle vector. The shuttle vector may be a plasmid

such as, pMK4, or pYL112Δ119. An expression vector may also be used, the expression vector is a plasmid with a very strong promoter, such as the following very strong promoters: p*Trc*99A, pDR540, or pET-21(+). In this nomenclature pTRC99A would be the name of the plasmid. Each plasmid used for expression of proteins has a unique promoter as follows: pTRC99A (trc promoter), pDR540 (tac promoter), pET-21(+) (T7 promoter).

Examples of plasmids would be a plasmid named pRODCAP18 comprising the cloned *rodC* gene, placed into the cloning vector, pUC18, the plasmid named pMKRODC comprising the the cloned *rodC* gene, placed into the shuttle vector pMK4, the plasmid where the plasmid was created from a *rodC* gene excised from a pRODCAP18 plasmid, the plasmid selected from the plasmids named pBSRODC1 or pBSRODC1, comprising the *rodC* gene, placed into an expression vector with a strong promoter that is p*Trc*99A and plasmids created where the *rodC* gene is excised from the pMKRODC plasmid.

These plasmids may be used to create transformed bacterial cells and collections of mutant cells and plasmids may be easily created. So there are further descriptions of a bacterial cell transformed with the various disclosed plasmids and a bacterial cell that is an *E.coli* cell, and an *E.coli* cell variously transformed that is of type DH10B.

New and novel assays are also disclosed and a most important assay disclosed herein does NOT demand the newly discovered DNA and protein although in some embodiments they are required. This invention comprises: A method of measuring the activity of the TAP enzyme comprising combining CDP-glycerol plus H_2O or water plus TAP enzyme plus lipoteichoic acid and measuring the amount of lipoteichoic acid formed. In one embodiment the CDP-glycerol is radioactive CDP-glycerol, in one embodiment the activity of the TAP enzyme comprises combining radioactive CDP-glycerol plus H_2O plus TAP enzyme plus lipoteichoic acid plus strepavidin SPA beads and a suitable lectin such as a wheat germ agglutinin and measuring the amount of radioactive lipoteichoic acid formed as indicated by measuring the lectin bound to the SPA bead. In all these embodiments the radioactive CDP-glycerol cna be made from [^{3}H]glycerol-3-phosphate (a.k.a. [^{3}H]glycerophosphate). A preferred method of practicing any of these assays is to treat the lipoteichoic acid to remove the alanine residues before using it in the assays, that is, before combining with the other ingredients, the lipoteichoic acid is treated to remove alanine. These assays may be used to measure the activity of TAP enzyme when it is from an impure preparation or the methods may be used

where the TAP enzyme is the enzyme disclosed in Sequence Chart 1 or 2, or Sequence ID listing number 2. The assays herein may be configured into kits for ease of application.

Also disclosed is a method of using lipoteichoic acid as a substrate for the enzymatic reaction catalyzed by the TAP protein. Lipoteichoic acid, unlike teichoic acid, is commercially available and thus makes an excellent substrate. The lipoteichoic acid can serve as an acceptor of CDP[^{3}H]glycerol. The TAP protein can be obtained from crude sources or extracts, preferably the lipoteichoic acid is prepared from *B. subtilis*, *S. aureus*, or *E. faecalis*, or it can be the TAP protein described in Sequence Charts 1 and 2 and Sequence Listing I.D. no. 2, or a protein having at least about 70% homology to that protein.

A diagnostic kit utilizing the TAP enzyme and CDPglycerol to detect and monitor disease caused by gram positive bacteria can be created using the information disclosed herein. Following appropriate instructions from such a kit, a portion of the biological sample which is thought to contain lipoteichoic acid could be added to TAP and CDPglycerol, incubated for an hour or so, and the transfer of glycerol-3-phosphate from CDPglycerol to lipoteichoic acid present in the sample could be detected using the precipiation assay described below under "Precipitation Assay."

Brief Description of the Drawings

Figure 1. SDSPAGE of TAP purification scheme. Lanes 1 and 7 are molecular weight markers; lane 2 is the soluble protein fraction from cells containing overexpressed TAP; lane 3 is the membrane from the vector pTrc99A control; lane 4 is the 2M NaCl membrane extract from cells overexpressing TAP; lane 5 is the High Q purified TAP; and lane 6 is the Superose 12 purified TAP.

Figure 2. Southern blot showing the DNA sequence identified as being homologous to the sequence disclosed in Sequence Chart 1 only from the bacteria *Staphylococcus aureus*.

Additional Description of the Invention

This invention discloses a recombinant form of Teichoic Acid Polymerase (or TAP), also known as CDP-Glycerol:Poly(glycerophosphate) Glycerophosphotransferase, its amino acid sequence and the DNA that codes for this enzyme. In addition, vectors, plasmids, probes and cells expressing this enzyme and assays incorporating the enzyme and disclosed herein, and all useful in some stage of discovery of new antibiotics, or the monitoring of disease states.

The genes responsible for cell wall teichoic acid synthesis in *B. subtilis* have

been located in an operon on the chromosome, C. Mauel, M.Young, D. Karamata, "Genes concerned with synthesis of poly(glycerol phosphate), the essential teichoic acid in *Bacillus subtilis* strain 168, are organized in two divergent transcription units" *J. Gen. Microbiol.* (1991) 137:929-941. The *tag* genes, A-F, have all been sequenced, *Id.*, but only the *tagD* gene protein product has been purified and characterized. Y.S. Park, T.D. Sweitzer, J.E. Kison, C. Kent. "Expression, purification, and characterization of CTP:Glycerol-3-phosphate cytidyltransferase from *Bacillus subtilis*." *J. Biol. Chem.* (1993) 268:16648-16654.

RodC or *rodc*, also called *TagF, Tagf, tagf* or *tagF* codes for CDP-Glycerol:Poly(glycerolphosphate) Glycerophosphotransferase, which will be referred to as Teichoic Acid Polymerase, or more frequently, TAP herein. This enzyme, TAP, catalyzes the polymerization of the polyglycerolphosphate backbone of teichoic acid by linking together the glycerolphosphate moiety of CDP-glycerol into 1,3 phosphodiester linkages. Attempts to isolate deletion mutants of *rodC* have been unsuccessful, see, C. Mauel, M. Young, P. Margot, D. Karamata "The essential nature of teichoic acids in *Bacillus subtilis* as revealed by insertional mutagenesis" *Mol. Gen. Genet.* (1991) 215:388-394 and A.L.Honeyman, G.C. Stewart "Identification of the protein encoded by *rodC*, a cell division gene from *Bacillus subtilis*" *Mol. Microbiol.* (1988) 2:735-741; however, there is one temperature sensitive mutant RODC113 available for study. This strain has a point mutation in the *rodC* gene which decreases enzyme activity sufficiently at 55°C to stop growth. See, Honeyman AL, Stewart GC. "The nucleotide sequence of the *rodC* operon of *Bacillus subtilis*." *Mol. Microbiol.* (1989) vol. 3 pp.1257-1268.

The inability of deletion mutants of *rodC* to survive and the fact that TAP is essential to the viability of *B. subtilis* suggest the temperature sensitive *rodC* enzyme and mutants described herein are particularly valuable. The cloning, sequencing, and partial purification of a novel form of TAP are described below.

Cloning theory. Previous investigators have attempted to identify and clone the *rodc* gene and protein. See, A.L.Honeyman, G.C. Stewart, "Identification of the protein encoded by *rodC*, a cell division gene from *Bacillus subtilis*" *Mol. Microbiol.* (1988) 2:735-741 and A.L.Honeyman, G.C. Stewart, "The nucleotide sequence of the *rodC* operon of *Bacillus subtilis*. *Mol. Microbiol.* (1989) 3:1257-1268. These previous investigations, using the bacillus known as *Bacillus subtilis*, did not result in the successful production of the TAP enzyme. The previous investigations disclosed a different sequence than the open reading frame of the *rodc* gene disclosed here. The previous investigations disclosed a different protein, one that had a very different

size and one where no compositional analysis of the putative protein sequence was ever provided. The enzyme produced from the cDNA disclosed herein has never before been produced from expressed cDNA, nor has the shortened cDNA sequence disclosed here been reported as that responsible for the entire TAP protein. Previous disclosures reported a different, larger DNA sequence and a different, purely notional protein. The sequence of an active TAP enzyme has never been reported. However, the previously reported information was useful for the creation of probes, which were then used to discover and create the DNA.

In addition to creating the sequence from *Bacillus subtilis*, the authors herein have isolated DNA sequences from *Staphylococcus aureus* that arehomologous to the *tagF B. subtilis* residues.

Cloning and sequencing.

The *B. subtilis rodC* gene encoding TAP was amplified from chromosomal DNA using PCR. Sequencing revealed that *Taq* polymerase had performed a nucleotide misincorporation, resulting in a C to T transition at bp 1871. However, the resulting alanine to valine change in the expressed protein did not prevent the cloned gene from complementing a temperature sensitive defect in the *rodC* gene of *B. subtilis* strain RODC113.

Sequence Chart 1 shows that the DNA sequence of the PCR product cloned into pUC18 (pRODCAP18) matched the published *rodC* sequence exactly except for a C to T transition at bp 1871. The resulting point mutation changed an alanine in the wild-type TAP to valine. This mutant *rodC* gene was capable of complementing the *rodC* defect in the temperature sensitive *B. subtilis* strain RODC113 by allowing growth at the nonpermissive 55°C temperature.

The mutant *rodC* gene (*rodCCT*) was excised from pMKRODC as a 2.3 kb *Bam*HI fragment and cloned into the expression vector p*Trc*99A to form pBSRODC1. Induction of *E. coli* DH10B/pBSRODC1 cells with 5 mM IPTG resulted in the appearance of a ca. 85 kd band in the cell membrane preparation (**Figure 1, lane 4**). The vector control (p*Trc*99A) membrane preparation did not contain this band (**Figure 1, lane 3**), but the 100,000 x g cell extract supernatant from pBSRODC1 did show slightly less 85 kd protein than the membrane preparation (**compare lanes 2 and 4, Figure 1**). N-terminal sequence analysis of the blotted protein revealed that the 85 kd polypeptide amino acid sequence began with MIENTVIKC. **Sequence Chart 1** shows that this sequence corresponds to a 2163 bp open reading frame beginning with ATG at bp 100 and ending with TAA at bp 2263.

Cloning an homologous sequence from *Staphylococcus aureus*.

Chromosomal DNA from S. aureus was isolated and digested with restriction enzymes. The cut DNA was subjected to a Southern using the *B. subtilis tagF* gene (TAP producer) as a DNA probe. This experiment showed that *S. aureus* has DNA sequences that are highly homologous to the *B. subtilis tagF* gene.

Chromosomal DNA from S. aureus (reference = American Type Culture Collection [ATCC] 29213) was isolated and digested with the restriction enzymes *EcoRI* and *HindIII*. The DNA digests were separated by electrophoresis on a 1% agarose gel and blotted to a Nylon membrane generally following the method of Southern (Southern, E.M. 1975. "Detection of specific sequences among DNA fragments separated by gel electrophoresis," *J. Mol. Biol.* vol. 98, pp. 503, incorporated by reference.) A DNA probe was prepared using the method of nick translation and a 2.3 kb segment of the cloned *tagF* gene from *B. subtilis*. The *S. aureus* digests were hybridized to the *tagF* probe and several bands were identified which had homology to the cloned *B. subtilis tagF* gene. For example, the EcoRI digest of *S. aureus* chromosomal DNA yielded DNA fragments that were 7.0 kb, 5 kb, and 4.2 kb in size which hybridized well to the *B. subtilis tagF* gene. In addition, a *HindIII* digest of the same *S. aureus* chromosomal DNA yielded three homologous bands that migrated at 4.5 kb, 3.3 kb and 2.8 kb, respectively. As judged by visual inspection of the autoradiograms, the 4.5 kb HindIII fragment was much less homologous than either the 3.3 or 2.8 kb bands. This Southern analysis has identified *tagF* homologs in *S.aureus* that hybridize to the *tagF* gene of *B. subtilis*. These homologous *S. aureus* sequences represent genes coding for teichoic acid synthesis.

Production and Purification of the TAP enzyme.

TAP was overproduced under control of the *trc* promoter in *E. coli* DH10B cells. The protein was primarily located in the cell membrane, and salt extraction was used to initiate purification. TAP is associated with the membrane in *B. subtilis*, but the amino acid sequence does not indicate membrane spanning regions. It appears that TAP is loosely associated with the cell membrane.

Purification of TAP was hindered by the association of DNA with the enzyme preparation. Gel filtration chromatography did not improve purification, indicating that nucleic acid and protein formed a tight complex. Incubation of this preparation with Benzonase released nucleic acid fragments, but did not alter the size of the complex significantly. Future purification will require the addition of Benzonase early in purification in order to prevent the complex from forming.

Despite the nucleic acid problem, TAP was purified four-fold from the

membrane preparation. The enzyme was stable for two weeks when stored in ice. Though TAP synthesizes cell wall teichoic acid *in situ*, lipoteichoic acid from either *B. subtilis*, *S. aureus*, or *E. faecalis* could serve as an acceptor of CDP[^{3}H]glycerol. The availability of a commercial source of lipoteichoic acid will allow the development of the TAP assay for a high volume screen which could lead to the discovery of TAP inhibitors. It appears that TAP recognizes the polyglycerol-phosphate backbone of either cell wall teichoic acid or lipoteichoic acid and largely ignores the proximal portion of either polymer.

The biosynthetic pathway for teichoic acid has been established for many years, yet the exact function of this anionic polymer has never been determined. One report describes the use of teichoic acid as a reserve phosphate source in which gram positive bacteria draw upon the glycerolphosphate when phosphate levels in the environment are low (Grant WD. "Cell wall teichoic acid as a reserve phosphate source in *Bacillus subtilis*" *J Bacteriol* (1979) vol.137, pp. 35-43, incorporated by reference). While this role for teichoic acid cannot be disputed, the fact that *B. subtilis* cannot survive in the absence of teichoic acid synthesis under conditions of high phosphate levels (Mauel C, Young M, Margot P, Karamata D. "The essential nature of teichoic acids in *Bacillus subtilis* as revealed by insertional mutagenesis" *Mol Gen Genet* (1991) vol. 215, pp. 388-394, incorporated by reference) indicate that a more essential role is likely. Some reports point to the ability of teichoic acid to chelate divalent cations (Fischer, W. "Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*" *Med. Microbiol. Immunol.* (1994) vol.183, pp. 61-76, incorporated by reference), but lipoteichoic acid would presumably chelate in the absence of cell wall teichoic acid. It is far more likely that the essential nature of teichoic acid is in maintaining the structural integrity of the cell wall, due to the covalent attachment to peptidoglycan (**Technical Chart 1**). Given the information disclosed herein it would be obvious to one skilled in the art to randomly mutate the cloned *rodC* gene, integrate the mutated gene back into the chromosome, and produce a pool of TAP mutants which can be used to study the effects of teichoic acid on gram positive cell wall integrity.

Partial purification of TAP.

The enzymatic activity of TAP was assayed using CDP[^{3}H]glycerol as the glycerolphosphate donor and *B. subtilis* lipoteichoic acid as the acceptor. If active, the recombinant TAP enzyme should lengthen lipoteichoic acid with radioactive glycerolphosphate monomers, producing acid precipitable radioactivity. Preliminary experiments demonstrated that the overexpressed TAP was active, therefore a

purification method was initiated. Extraction of TAP from the *E. coli* membrane with 2 M NaCl produced an active TAP preparation that could be separated from the cell membrane by ultracentrifugation. Dialysis of the TAP membrane extract against Buffer A (see Material and Methods) produced the protein pattern shown in **Figure 1**, lane 4.

Ten mg of the dialyzed membrane extract was placed in an ion-exhange column. Equilibration of the column with Buffer A containing 50 mM NaCl resulted in a small amount of TAP activity passing through the column in fractions 2-4, but the majority of activity eluted at the end of the 0.05 M to 0.5 M NaCl gradient. **Table 1** shows that High Q chromatography resulted in a four-fold purification of TAP, and SDSPAGE analysis of these pooled fractions demonstrated that TAP was enriched (**Figure 1**, lane 4). Gel filtration chromatography on Superose 12 did not increase the specific activity of the enzyme (data not shown) and this result is supported by the lack of purification evident in lane 5 of **Figure 1**. TAP eluted with the void volume (mol. wt. >200 kd) in the Superose 12 column, while it migrated as a ca. 400-600 kd protein using a TSK-400 gel filtration column. A spectrophotometric wavelength scan revealed that the sample contained a high amount of nucleic acid which presumably forms a high molecular weight complex with the proteins in the sample, including TAP.

Applications and uses of the TAP enzyme, lipoteichoic acid as a substrate, and the expressed cloned cDNA sequence.

Enzymatic Synthesis of Polyglycerolphosphate (Teichoic Acid) by TAP and alternative substrates for the TAP enzyme.

TAP catalyzes the synthesis of the polyglycerolphosphate backbone of cell wall teichoic acid in *B. subtilis*, and this polymer is covalently attached to peptidoglycan (**Technical Chart 1**). Lipoteichoic acid is a structurally related polymer that is anchored to the cell membrane of gram positive bacteria by the fatty acyl side chains of a phospholipid moiety (**Technical Chart 2**). Both lipoteichoic acid and cell wall teichoic acid share the same polyglycerolphosphate backbone but there is evidence that TAP does not synthesize lipoteichoic acid *in situ* (Fischer, W. "Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*" *Med. Microbiol. Immunol.* (1994) vol. 183, pp. 61-76). Herein, we present data that shows that lipoteichoic acid can serve as an alternate substrate for TAP. This is an important discovery, both because lipoteichoic acid is available commercially and cell wall teichoic acid is not, and because tests have suggested that soluble teichoic acid does not serve as a suitable substrate for TAP. This discovery now makes it

possible to develop mechanistic screens for TAP inhibitors.

Assay Conditions.

Several assays may be constructed using the TAP enzyme. Precipitation and SPA are two examples. Modification (alanine removal) of lipoteichoic acid resulted in improved activity of the recombinant TAP enzyme. Alanine ester was removed from lipoteichoic acid by resuspending 1 mg. in 0.1M Tris-HCL buffer (pH 8.0) for 24 hr. at 37^0 C. Free alanine was removed by dialysis in 3500 dalton cutoff membrane agains deionized water (Fischer, W., H.U. Koch, P. Rosel, and F. Fiedler "Alanine ester-containing native lipoteichoic acids do not act as lipoteichoic acid carrier" *J. Biol. Chem.*, (1980) vol. 255, pp. 4557-4562, incorporated by reference.

Precipitation Assay

The method of Burger and Glaser was generally followed. Burger MM, Glaser L. "The synthesis of teichoic acids" *J. Biol. Chem.* (1964) vol. 239, pp. 3168-3177, incorporated by reference. A typical assay contained 1-100 µl of enzyme, 10 µl of lipoteichoic acid (1 mg/ml *B. subtilis* lipoteichoic acid [Sigma] in water), 25 µl of CDP[^{3}H]glycerol (10 mM CDPglycerol Specific Activity 8.8 µCi/µMole), and enough Buffer A to bring the total volume to 250 µl. The reaction was incubated at 37°C for one hour, mixed with 80 µl of 3N perchloric acid, and placed on ice for 5 min. The acid treated sample was spotted on a GF/C filter and washed with 4 x 5 ml of 0.15 N perchloric acid before liquid scintillation counting. Control reactions lacking either lipoteichoic acid or CDP[^{3}H]glycerol were included as negative controls.

Preliminary experiments demonstrated that incubation of lipoteichoic acid and CDP[^{3}H]glycerol with TAP resulted in the formation of an acid precipitable material. When this material was analyzed by cellulose thin-layer chromatography, it remained at the origin, indicating that a high molecular weight compound had been formed in the TAP assay. Hydrolysis of this product at 100°C in the presence of 1 N HCl resulted in the formation of degradation products which comigrated with glycerol and glycerol-3-phosphate. The chromatography profile matches that which has been reported for the acid catalyzed degradation of polyglycerol-phosphate (Burger MM, Glaser L. "The synthesis of Teichoic Acids" *J. Biol. Chem.* (1964), vol. 239, pp. 3168-3177, incorporated by reference) the data demonstrate that lipoteichoic acid can serve as an acceptor for the transfer of [^{3}H]glycerol-3-phosphate from CDP[^{3}H]glycerol in the TAP catalyzed reaction, thereby lengthening the polyglycerol-phosphate chain. **Tabl 2** shows that commercial lipoteichoic acid preparations from *S. aureus* and *S. faecalis* can also serve as acceptors for the

transfer of [^{3}H]glycerol-3-phosphate from CDP[^{3}H]glycerol.

Scintillation Proximity Assay (or SPA)

This assay is based on the ability of lectins such as wheat germ agglutinin (WGA) and concanavalin A (conA) to bind the sugar moieties present on lipoteichoic acids isolated from a variety of gram positive bacteria. For example, the enzyme can be mixed with buffer, [^{3}H]CDP-glycerol, and 10 µg of *Enterococcus faecalis* lipoteichoic acid as described for the precipitation assay above. After incubating at 37°C for 1 hour, strepavidin SPA beads (Amersham) containing biotinylated concanavilin A are added to the assay and the entire mix is incubated at room temperature for 30 min. in a 96 well plate. The conA::SPA bead conjugate will bind the radioactive lipoteichoic acid formed in the assay and the activity of the enzyme can be quantitated using a counter such as a Packard Top Counter. A variety of lipoteichoic acids can serve as substrates and the appropriate lectin can be bound to a SPA bead. For example, the glucose moieties present on the lipoteichoic acids of *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus hirae* can be bound to SPA beads containing conA. The cell wall teichoic and lipoteichoic acids of *Staphylococcus aureus* containing N-acetylglucosamine residues can be bound to WGA beads.

Alternative uses for the discovery that lipoteichoic acid can be used as a substrate for the TAP enzyme.

The discovery that lipoteichoic acid can be used as a substrate for the TAP enzyme suggests other practical applications. One obvious application of this discovery will be the creation of kits and diagnostic devises useful for the monitoring and management of disease states caused or influenced by gram positive bacteria.

Since lipoteichoic acid serves as a substrate for TAP, in that TAP will extend the lipoteichoic acid chain by adding glycerol-3-phosphate residues from CDPglycerol, TAP could therefore be used to detect the presence of lipoteichoic acid in biological samples including blood and other bodily fluids. For example, a portion of the biological sample which is thought to contain lipoteichoic acid could be added to TAP and CDPglycerol, incubated for an hour or so, and the transfer of glycerol-3-phosphate from CDPglycerol to lipoteichoic acid present in the sample could be detected using the precipiation assay described under "Precipitation Assay."

Potential uses of TAP could therefore include the diagnosis of bacterial infection in which bacteria release lipoteichoic acid into body fluids. TAP can be used to detect lipoteichoic acid in body fluids. Antibodies which target lipoteichoic acid are currently used for lipoteichoic acid detection in clinical samples, but the

discovery disclosed here makes it possible for the TAP enzyme to be used to perform the same function.

Materials and Methods

One skilled in the art should be able to reproduce and practice this invention with the information provided above, the materials and methods below are provided to further illuminate the invention and should not be considered limiting in any way.

Cloning *rodC* From *B. subtilis*

The published sequence of *rodC* was utilized to clone the gene from the *B. subtilis* chromosome using PCR, according to the following:. The primers:

C1A (5'-TTCAGGATCCTTCTCTTGGAGG GTCACGGAAATAAAAG-3'), SEQ. ID NO. 3 and

C2A (5'-ATTTGGATCCCCTAAATTATTCAGCTTTAAATAC-3') SEQ. ID NO. 4

hybridized to sequences -35 to -8 bases upstream of the putative translational start site and -15 to +9 from the stop site, respectively. Primer sequences are reproduced in Sequence Chart 3 and sequence identification numbers 3 and 4. Amplification using a program of 94°C for 45 sec/48°C for 45 sec/ and 72°C for 2 min (30 cycles) resulted in the production of a 2.3 kb product which yielded *EcoRI* fragments of 1.5 and 0.8 kb, characteristic of *rodC*. The *Bam*HI sites engineered into the primers C1A and C2A allowed for the cloning of *rodC* into pUC18, yielding pRODCAP18. Any other commonly available cloning vector could be used in place of pUC, including such vectors as pUC's, pUC18, pUC19, pBR322, and many other commonly available plasmids.

Complementation of the *B. subtilis rodC* mutant RODC113

The *rodC* gene from pRODCAP18 was excised as a 2.3 kb *Bam*HI fragment and ligated into the *Bam*HI site of the *E. coli*/gram positive shuttle vector pMK4 to produce pMKRODC. The pMK4 plasmid was selected because it reproduces in both gram negative bacteria like *E.coli* and it reproduces in gram positive bacteria like *B. subtilis*. Any shuttle vector of this type should be suitable. pMKRODC was electroporated into the temperature sensitive *B. subtilis rodC* mutant RODC113 using an standard methods. The cells were plated on LB/chloramphenicol and incubated at 55°C.

Sequencing

The *rodC* gene present in pRODCAP18 was sequenced using deltaTaq cycle sequencing (Amersham®) and [^{35}S]dATP direct incorporation.

Overexpression of TAP

In order to achieve overexpression of TAP, the *rodC* gene was excised from pMKRODC as a 2.3 kb *Bam*HI fragment and ligated into the *Bam*HI site of the expression vector p*Trc*99A (Pharmacia) to form pBSRODC1. The expression vector p*Trc*99A was chosen here but any plasmid with a very strong promoter, such as p*Trc*99A, pDR540, or pET-21(+) should make a suitable expression plasmid. A second isolate containing *rodC* in the opposite orientation was designated pBSRODC2. In these examples pTRC99A would be the name of the plasmid. Each plasmid used for expression of proteins has a unique promoter as follows: pTRC99A (trc promoter), pDR540 (tac promoter), pET-21(+) (T7 promoter).

Overexpression and Partial Purification of TAP From *Escherichia coli*

A. Cell Growth and Lysis

DH10B (*E. coli*) cells transformed with pBSRODC1 were grown in 2 liters of 2X LB for 3 hr, induced with IPTG (5 mM) for 4 hr, harvested via centrifugation, and stored at -70°C. The cell pellet was resuspended in 5 ml of Buffer A (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, pH 7.5) and lysed by two passages through a French Pressure Cell at 10,000 psi. Note, 2X LB can be restated as double strength Lennox Broth. IPTG stands for isopropylthio-beta-D-galactoside.

B. Extraction of TAP From the Cell Membrane

The cell lysate was centrifuged at 5,000 x g for 15 min to remove unbroken cells, and the supernatant was centrifuged at 100,000 x g for one hour. The resulting membrane pellet was resuspended in 25 ml of Buffer A containing 2M NaCl and extracted for two hours on a rotary shaker at 4°C. The sample was then centrifuged at 100,000 x g for one hour to pellet the membrane, and the supernatant was dialyzed overnight against four liters of Buffer B (Buffer A containing 50 mM NaCl).

C. High Q Anion Exchange Chromatography

A 5 ml High Q column (BioRad®) was equilibrated with Buffer B using an Econosystem Automated Chromatography Unit (BioRad®). A portion of the 2M NaCl extract of the cell membrane representing 10 mg of total protein was applied to the column and unbound proteins were washed out with the same buffer. TAP was eluted from the column using a 0.05 to 0.5 M NaCl gradient and fractions containing TAP activity were pooled and concentrated using a Centriprep 30 ultrafiltration unit (Amicon®). The concentrated protein was dialyzed overnight against two liters of Buffer A.

TAP Enzyme Assay

The method of Burger and Glaser was generally followed. Burger MM, Glaser L. "The synthesis of teichoic acids" *J Biol Chem* (1964) vol. 239, pp. 3168-3177, incorporated by reference. A typical assay contained 1-100 µl of enzyme, 10 µl of lipoteichoic acid (1 mg/ml *B. subtilis* lipoteichoic acid [Sigma] in water), 25 µl of CDP[^{3}H]glycerol (10 mM CDPglycerol Specific Activity 8.8 µCi/µMole), and enough Buffer A to bring the total volume to 250 µl. The reaction was incubated at 37°C for one hour, mixed with 80 µl of 3N perchloric acid, and placed on ice for 5 min. The acid treated sample was spotted on a GF/C filter and washed with 4 x 5 ml of 0.15 N perchloric acid before liquid scintillation counting. Control reactions lacking either lipoteichoic acid or CDP[^{3}H]glycerol were included as negative controls.

Identification of the TAP Product as Polyglycerolphosphate

The TAP assay was performed as described above in a total volume of 1 ml by scaling up the appropriate reagents. After one hour at 37°C, the product of the reaction was precipitated with 3N perchloric acid and centrifuged at 14,000 x g for 15 min. The pellet was washed two times with 0.5 ml of 0.15 N perchloric acid and dried in a speed vac before resuspending in 0.1 ml of 0.3 M NH_4OH. At this point the soluble material represented 5.3 x 10^6 cpm per ml. Approximately 0.09 ml of this material was dried in the speed vac and subsequently resuspended in 0.1 ml of 1N HCl. This material was refluxed in a sealed vial at 100°C and samples were removed after 16 hr of hydrolysis for analysis by thin layer chromatography using cellulose plates and the following solvent systems: Ethanol - NH_4acetate, pH 7.5 (7.5:3) and n-propanol - ammonia - water (6:3:1). After developing the plates in the solvent, they were dried at room temperature and cut into 1 x 2 cm sections for liquid scintillation counting.

Additional disclosed embodiments of the invention

With this disclosure of the TAP sequence, random mutation of the cloned *rodC* gene may be constructed and integrated back into the chromosome thus producing a pool of TAP mutants which can be used to study the effect of teichoic acid on gram positive cell wall integrity.

Definitions

Words in this document should be given the meaning that one skilled in the art with give those words. Some examples of this follow.

BioRad® is the name of a biochemical supply company located in Hercules, California

Amicon® is the name of a biochemical supply company located in Beverly, Massachusetts.

h or hr is hour

homology and homologous sequences and residues are referred to in this document. In general, these terms have meanings generally accepted by one skilled in the art. The following definitions are also provided and should control and explain any scientific disagreement concerning the meaning of the terms. Here are two definitions of homology, one in reference to DNA, or nucleic acid sequences and one in reference to peptide or protein sequences. Nucleic acid homology definition: a nucleic acid sequence from one organism is X% homologous to that of a second organism when a gene from the second organism contains, at any point within the sequence, X nucleotide residues out of 100 which were identical to that of a similar gene of the first organism. For example, a nucleic acid sequence from S. aureus which is 70% homologous to that of the B. subtilus rodC gene would contain at any point within the sequence, 7 nucleotide residues out of 10 which were identical to that of the B. subtilis rodC gene. Peptide or protein homology: a peptide or protein, which is X % homologous to that of another peptide or protein, would contain, at any point within its amino acid sequence, X amino acid residues out of 100 which were either identical or similar to that of the first amino acid sequence. For example, a peptide or protein which is 70 % homologous to that of the B. subtilis TAP enzyme, would contain, at any point within its amino acid sequence, 7 amino acid residues out of 10 which were either identical or similar to that of the B. subtilis amino acid sequence. A similar amino acid is one that is of similar size, charge or hydrophilic property.

IPTG stands for isopropylthio-beta-D-galactoside.

m. or min. is minute

Cloning vectors are vectors such as pUC's pUC18, pUC19, pBR322, and many other commonly available plasmids.

A cloning vector may be a shuttle vector.

A shuttle vector is a plasmid that replicates in either gram negative or gram positive bacteria. Example shuttle vectors are pMK4, and pYL112Δ119.

An expression vector is a plasmid with a very strong promoter, such as p*Trc*99A, pDR540, or pET-21(+).

TABLE 1

TAP Enzymatic Activity in Purification Procedure.

Purification Step	Specific Activity nmoles/hr/mg protein
Membrane	4.7
2M NaCl Extract of Membrane	10.3
High Q Chromatography	39.9

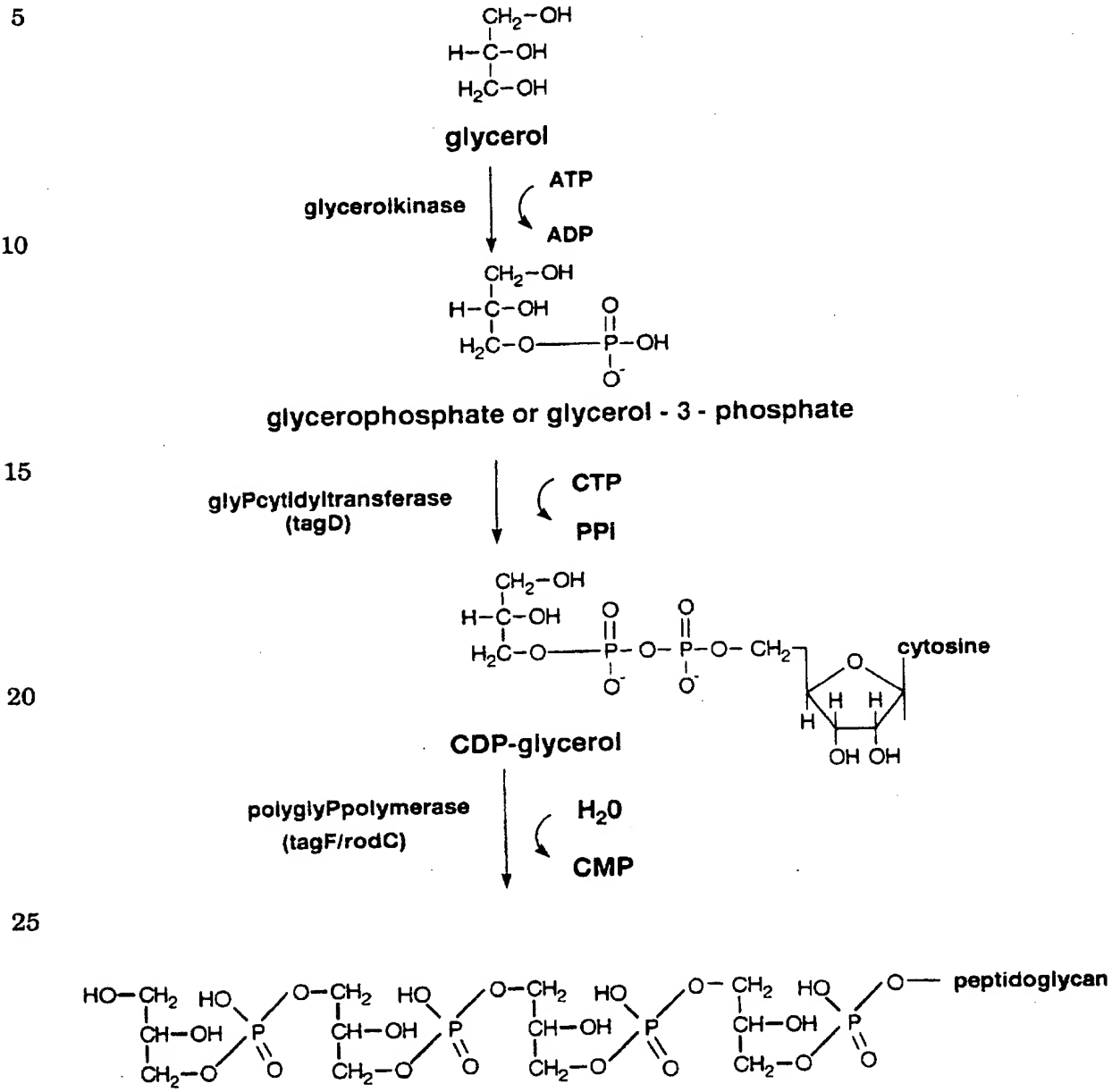
TABLE 2

Effect of Lipoteichoic Acid Source on TAP Activity.

Source of Lipoteichoic Acid	cpm of [^{3}H] glycerol Incorporated
Bacillus subtilis	155,930
Streptococcus faecalis	93,814
Staphylococcus aureus	15,632

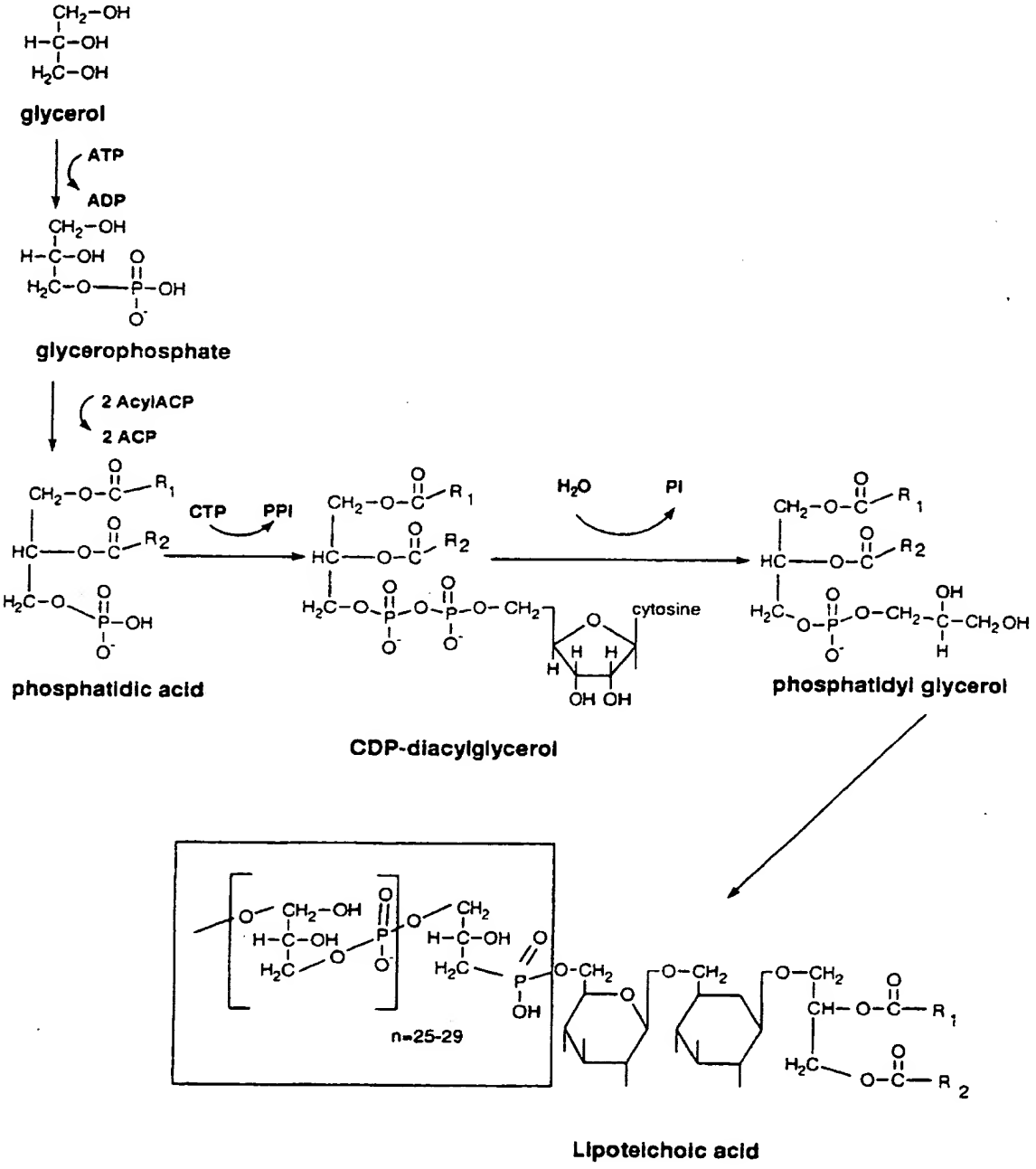
TECHNICAL CHART 1

Teichoic Acid Pathway in *B. subtilis*. Biosynthetic pathway for cell wall teichoic acid synthesis in *B. subtilis*. The polyglycerolphosphate polymer of teichoic acid is linked to peptidoglycan in gram positive bacteria.



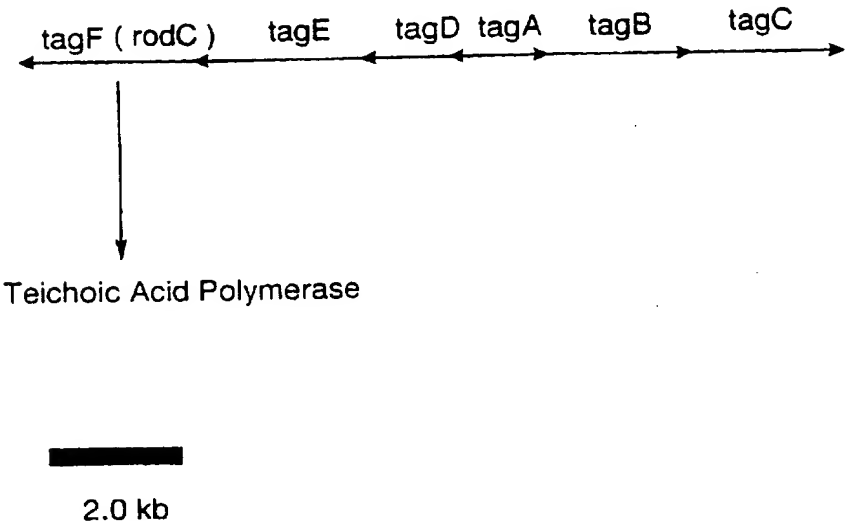
TECHNICAL CHART 2

Biosynthesis of Lipoteichoic Acid in *Staphylococcus aureus*. Biosynthetic pathway for lipoteichoic acid synthesis. The fatty acyl chains of lipoteichoic acid are embedded in the cell membrane and the polyglycerolphosphate backbone is oriented out towards the cell surface.



TECHNICAL CHART 3

B. subtilis teichoic acid biosynthetic operon showing the location of the *rodC* (*tagF*) gene which codes for teichoic acid polymerase.



SEQUENCE CHART 1

The DNA Sequence of the *rodC* gene from *B. subtilis*. Restriction sites are indicated. The translated protein sequence is also provided. This CHART also shows the mutation at position 1871, a "T for C" DNA substitution resulting in a "V for A" amino acid substitution. The DNA from this Sequence Chart 1 is listed as Sequence Identification number 1, SEQ. ID. NO. 1, (SEQ. ID. NO. 1 is the sequence without the mutation at position 1871). The protein from this Sequence Chart 1 is listed in Sequence Chart 2 and is listed as SEQ. ID. NO. 2. The numbers in the left margin in the Chart below indicate nucleic acid residues. The ATG shown below is the true start codon for the actual first amino acid of the isolated TAP enzyme. In practice, an upstream ribosome binding site is also required. The actual DNA sequence that was inserted into the plasmid is shown in Sequence Chart 4. Here this protein, sequence shown below, SEQ. ID. 2, and Sequence Chart 2, was actually expressed in *E. coli* using the plasmid pBSRODC1.

```
      ATGATTGAAAACACTGTGATT
    1 +---------+---------+
      M  I  E  N  T  V  I

      AAATGTATTTTGAAAAGCTTGAAAAACAATTTAGGAAGTCTTGAATTGTTAATCTCAATT
   22 ---------+---------+---------+---------+---------+---------+----------
      K  C  I  L  K  S  L  K  N  N  L  G  S  L  E  L  L  I  S  I

      GATTCAGAACACCAATTTTTAGAGGATTACCAGTTATTTTTAAAGCTGAAAGAGAGACGT
   82 ---------+---------+---------+---------+---------+---------+----------
      D  S  E  H  Q  F  L  E  D  Y  Q  L  F  L  K  L  K  E  R  R

      TCAGGAACGGAATCTGAATTTCCGCTTCAAAACACTGGCTCATTAGAGTATAAAACTGAG
  142 ---------+---------+---------+---------+---------+---------+----------
      S  G  T  E  S  E  F  P  L  Q  N  T  G  S  L  E  Y  K  T  E

      ATAAATGCTCATGTTTTGCCTATGCCTGTTGAAATGGGACAAACATATGATTTTTATGTC
  202 ---------+---------+---------+---------+---------+---------+----------
      I  N  A  H  V  L  P  M  P  V  E  M  G  Q  T  Y  D  F  Y  V

      GAATTTCGAAAAAAATATGAAGATGCGGAGCAGGAACCACTCTTGAAGCGTCTTTCTGCT
  262 ---------+---------+---------+---------+---------+---------+----------
      E  F  R  K  K  Y  E  D  A  E  Q  E  P  L  L  K  R  L  S  A

      GAAGTAAATTCAATTGAGCGCGCCTTTCATGTCGATCAAACCACAGAACTTTTGATTTTA
  322 ---------+---------+---------+---------+---------+---------+----------
      E  V  N  S  I  E  R  A  F  H  V  D  Q  T  T  E  L  L  I  L
```

```
    CCTTATACAACTGATAAAGGCAACTTTTCTATTAAGGTGAAAAGAGAGGCCAAAATCATC
382 ---------+---------+---------+---------+---------+---------+
    P  Y  T  T  D  K  G  N  F  S  I  K  V  K  R  E  A  K  I  I

    AGATTTGATCAAATCGAGATTAGCTCTGAAGAAATAAGCATAACAGGTTATGCGGGGTAC
442 ---------+---------+---------+---------+---------+---------+
    R  F  D  Q  I  E  I  S  S  E  E  I  S  I  T  G  Y  A  G  Y

    CTGAGTTCCGAAAATCAATATCGGATAAAAAACTTGAACCTTATTTTAAAAAAGGGTGGA
502 ---------+---------+---------+---------+---------+---------+
    L  S  S  E  N  Q  Y  R  I  K  N  L  N  L  I  L  K  K  G  G

    GAAACACCTATTGAGGAAAAATTTCCAATCAAGCTAGAAAGAAAAACACATGGCCTGGAA
562 ---------+---------+---------+---------+---------+---------+
    E  T  P  I  E  E  K  F  P  I  K  L  E  R  K  T  H  G  L  E

    AACATGAGAGCAGATGGTTTTGTTCCGGAACTGTATGATTTTGAAGTGAAAGTGCCTTTG
622 ---------+---------+---------+---------+---------+---------+
    N  M  R  A  D  G  F  V  P  E  L  Y  D  F  E  V  K  V  P  L

    AAAGAAATTCCTTTCTCAAATGAAAAACGTTATGTTTATCGTCTTTTTATGGAGTATATA
682 ---------+---------+---------+---------+---------+---------+
    K  E  I  P  F  S  N  E  K  R  Y  V  Y  R  L  F  M  E  Y  I

    TGCAATGACGATGAAGGAACGGATATTCAGTTCAACAGCACTGCTCTTGTTTTAGGAGAT
742 ---------+---------+---------+---------+---------+---------+
    C  N  D  D  E  G  T  D  I  Q  F  N  S  T  A  L  V  L  G  D

    CGAAAAAACAAATTAAAAGGATTAGTAAGTATTATTAAAACAAACAACGCACCAGTTCGT
802 ---------+---------+---------+---------+---------+---------+
    R  K  N  K  L  K  G  L  V  S  I  I  K  T  N  N  A  P  V  R

    TATGAAGTCTTTAAGAAAAAGAAAAAGCAGACTCTAGGTATCAGAGTAAACGACTATAGC
862 ---------+---------+---------+---------+---------+---------+
    Y  E  V  F  K  K  K  K  K  Q  T  L  G  I  R  V  N  D  Y  S

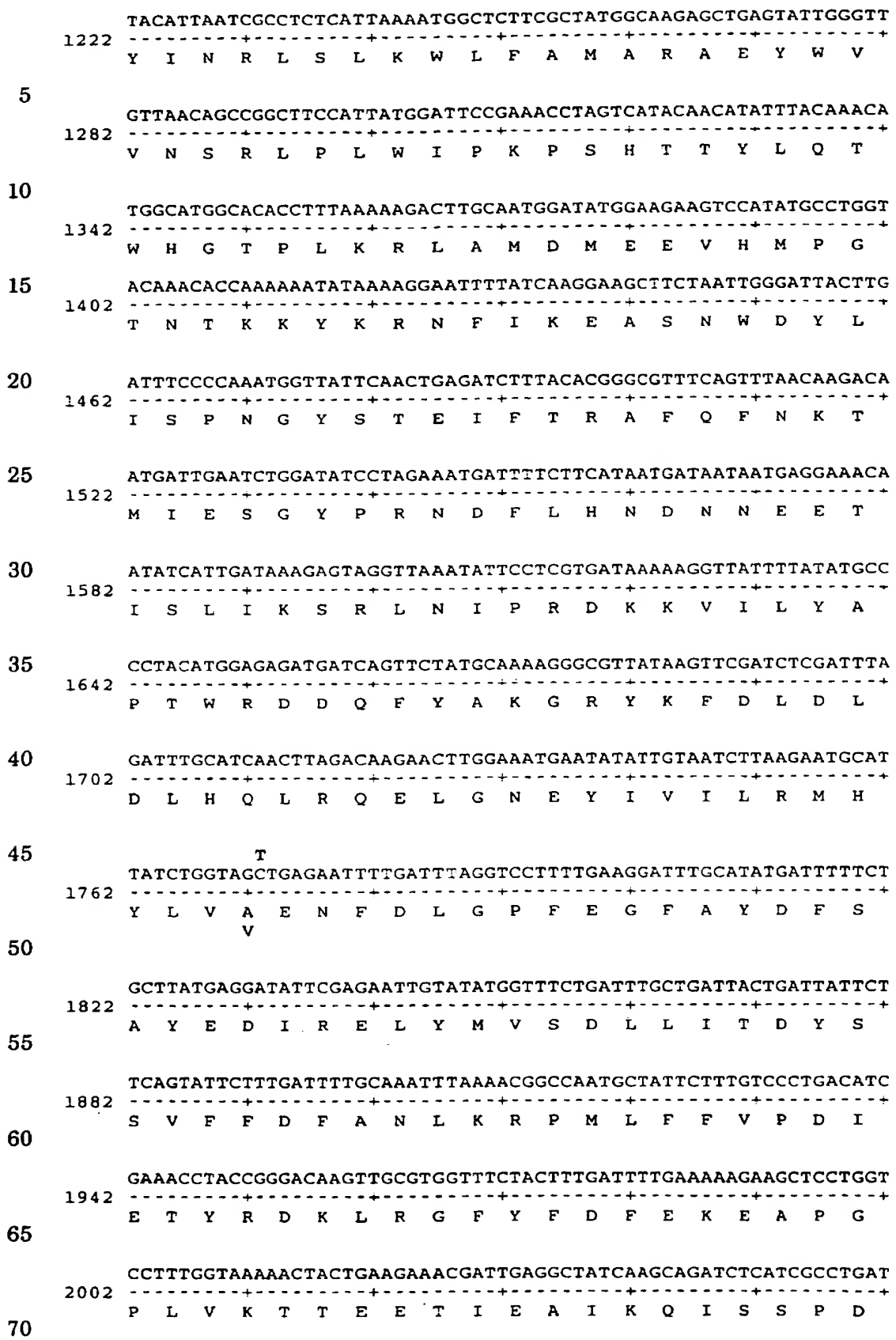
    CTGAAAACAAGGATGAAATACTTTATTAAAGGAAAGAAGAAGAGATTAGTATCAAAAATA
922 ---------+---------+---------+---------+---------+---------+
    L  K  T  R  M  K  Y  F  I  K  G  K  K  K  R  L  V  S  K  I

    AAAAAGATCACAAAAATGAGAAACAAGTTAATCACTAAAACATACAAATCTCTATTCATG
982 ---------+---------+---------+---------+---------+---------+
    K  K  I  T  K  M  R  N  K  L  I  T  K  T  Y  K  S  L  F  M

    ATGGCTAGCAGAATGCCAGTTAAAAGGAAAACAGTCATTTTTGAAAGTTTTAATGGGAAA
1042---------+---------+---------+---------+---------+---------+
    M  A  S  R  M  P  V  K  R  K  T  V  I  F  E  S  F  N  G  K

    CAATACAGTTGTAATCCGAGAGCGATTTACGAATATATGCGGGAAAACCACCCTGAGTAT
1102---------+---------+---------+---------+---------+---------+
    Q  Y  S  C  N  P  R  A  I  Y  E  Y  M  R  E  N  H  P  E  Y

    AAAATGTATTGGAGTGTAAATAAACAATATTCAGCGCCTTTTGATGAAAAGGGAATTCCT
1162---------+---------+---------+---------+---------+---------+
    K  M  Y  W  S  V  N  K  Q  Y  S  A  P  F  D  E  K  G  I  P
```



```
     TATAAGCTTCCGGTTTCTTTTGGTCCTTTCTATGATAAGTTTTGCTATTTAGAGTCAGGA
2064 ---------+---------+---------+---------+---------+---------+
     Y  K  L  P  V  S  F  G  P  F  Y  D  K  F  C  Y  L  E  S  G

     CGTTCATCTGAAAAGGTTGTTAATACTGTATTTAAAGCTGAATAATTTAGGGGATCCAAAT
2122 ---------+---------+---------+---------+---------+---------+
     R  S  S  E  K  V  V  N  T  V  F  K  A  E  *
```

SEQUENCE CHART 2

The amino acids, or protein from SEQUENCE CHART 1, including the single amino acid mutation, shown below. The amino acids from this Sequence Chart 2 are listed in as SEQuence IDentification Number 2 (SEQ. I.D. NO. 2 is the sequence without the mutation at position 616).

1	M	I	E	N	T	V	I												
8	K	C	I	L	K	S	L	K	N	N	L	G	S	L	E	L	L	I	S	I
28	D	S	E	H	Q	F	L	E	D	Y	Q	L	F	L	K	L	K	E	R	R
48	S	G	T	E	S	E	F	P	L	Q	N	T	G	S	L	E	Y	K	T	E
68	I	N	A	H	V	L	P	M	P	V	E	M	G	Q	T	Y	D	F	Y	V
88	E	F	R	K	K	Y	E	D	A	E	Q	E	P	L	L	K	R	L	S	A
108	E	V	N	S	I	E	R	A	F	H	V	D	Q	T	T	E	L	L	I	L
128	P	Y	T	T	D	K	G	N	F	S	I	K	V	K	R	E	A	K	I	I
148	R	F	D	Q	I	E	I	S	S	E	E	I	S	I	T	G	Y	A	G	Y
168	L	S	S	E	N	Q	Y	R	I	K	N	L	N	L	I	L	K	K	G	G
188	E	T	P	I	E	E	K	F	P	I	K	L	E	R	K	T	H	G	L	E
208	N	M	R	A	D	G	F	V	P	E	L	Y	D	F	E	V	K	V	P	L
228	K	E	I	P	F	S	N	E	K	R	Y	V	Y	R	L	F	M	E	Y	I
248	C	N	D	D	E	G	T	D	I	Q	F	N	S	T	A	L	V	L	G	D
268	R	K	N	K	L	K	G	L	V	S	I	I	K	T	N	N	A	P	V	R
288	Y	E	V	F	K	K	K	K	K	Q	T	L	G	I	R	V	N	D	Y	S
308	L	K	T	R	M	K	Y	F	I	K	G	K	K	K	R	L	V	S	K	I
328	K	K	I	T	K	M	R	N	K	L	I	T	K	T	Y	K	S	L	F	M
348	M	A	S	R	M	P	V	K	R	K	T	V	I	F	E	S	F	N	G	K
368	Q	Y	S	C	N	P	R	A	I	Y	E	Y	M	R	E	N	H	P	E	Y
388	K	M	Y	W	S	V	N	K	Q	Y	S	A	P	F	D	E	K	G	I	P
408	Y	I	N	R	L	S	L	K	W	L	F	A	M	A	R	A	E	Y	W	V
428	V	N	S	R	L	P	L	W	I	P	K	P	S	H	T	T	Y	L	Q	T
448	W	H	G	T	P	L	K	R	L	A	M	D	M	E	E	V	H	M	P	G
468	T	N	T	K	K	Y	K	R	N	F	I	K	E	A	S	N	W	D	Y	L
488	I	S	P	N	G	Y	S	T	E	I	F	T	R	A	F	Q	F	N	K	T
508	M	I	E	S	G	Y	P	R	N	D	F	L	H	N	D	N	N	E	E	T
528	I	S	L	I	K	S	R	L	N	I	P	R	D	K	K	V	I	L	Y	A
548	P	T	W	R	D	D	Q	F	Y	A	K	G	R	Y	K	F	D	L	D	L
568	D	L	H	Q	L	R	Q	E	L	G	N	E	Y	I	V	I	L	R	M	H

588	Y	L	V	A V	E	N	F	D	L	G	P	F	E	G	F	A	Y	D	F	S
608	A	Y	E	D	I	R	E	L	Y	M	V	S	D	L	L	I	T	D	Y	S
628	S	V	F	F	D	F	A	N	L	K	R	P	M	L	F	F	V	P	D	I
648	E	T	Y	R	D	K	L	R	G	F	Y	F	D	F	E	K	E	A	P	G
668	P	L	V	K	T	T	E	E	T	I	E	A	I	K	Q	I	S	S	P	D
688	Y	K	L	P	V	S	F	G	P	F	Y	D	K	F	C	Y	L	E	S	G
708	R	S	S	E	K	V	V	N	T	V	F	K	A	E	-	721				

SEQUENCE CHART 3

The following two primers were used to clone *rodC* from *B. subtilits*.

C1A is 5'-TTCAGGATCCTTCTCTTGGAGG GTCACGGAAATAAAAG-3', this is sequence I.D. number 3.

And C2A is 5'-ATTTGGATCCCCTAAATTATTCAGCTTTAAATAC-3', this is sequence I.D. number 4.

SEQUENCE CHART 4

The DNA Sequence of the *rodC* gene from *B. subtilis*. Restriction sites are indicated. The translated protein sequence is also provided. This CHART also shows the mutation at position 1871, a "T for C" DNA substitution resulting in a "V for A" amino acid substitution. The DNA from this Sequence Chart 4 is listed as Sequence Identification number 5 (seq. I.D. no.5 is the sequence without the mutation at position 1871). The protein only from this Sequence Chart 4 is listed in Sequence Chart 5 and is listed as SEQ. ID. NO. 6. The numbers in the left margin in the Chart below indicate nucleic acid residues. The ATG at nucleic acid residue 25-27 corresponds to the methionine translation start site predicted through computer analysis by reference to Honeyman and Stewart. The ATG underlined in nucleic acid residues 100-102 corresponds to the methionine (underlined and bold M) which is the actual first amino acid of the isolated TAP enzyme. Note, the actual DNA sequence, (with restriction sites noted), that was inserted into the plasmid is shown here and it includes an upstream ribosome binding site that is downstream from the putative start codon at position 25-27. The actual ribosome binding site is at position, 83-87. The actual ribosome binding site is apparently AGGAG, other ribosome binding sites could be engineered such as in AGGAGA site.

```
          BamHI
            |
25        TTTTGGATCCAAGGAAGAGAGTTAATGTCCTTAGTAGTTGACACTAATAAAAGGAAGCAA
        1 ---------+---------+---------+---------+---------+---------+
                                      M  S  L  V  V  D  T  N  K  R  K  Q

          AAAGGAAAGAGCTTTTATACAGAGGAGCAGAAAAAAGTAATGATTGAAAACACTGTGATT
30      61 ---------+---------+---------+---------+---------+---------+
          K  G  K  S  F  Y  T  E  E  Q  K  K  V  M  I  E  N  T  V  I
                     HindIII
                        |
          AAATGTATTTTGAAAAGCTTGAAAAACAATTTAGGAAGTCTTGAATTGTTAATCTCAATT
35     121 ---------+---------+---------+---------+---------+---------+
          K  C  I  L  K  S  L  K  N  N  L  G  S  L  E  L  L  I  S  I

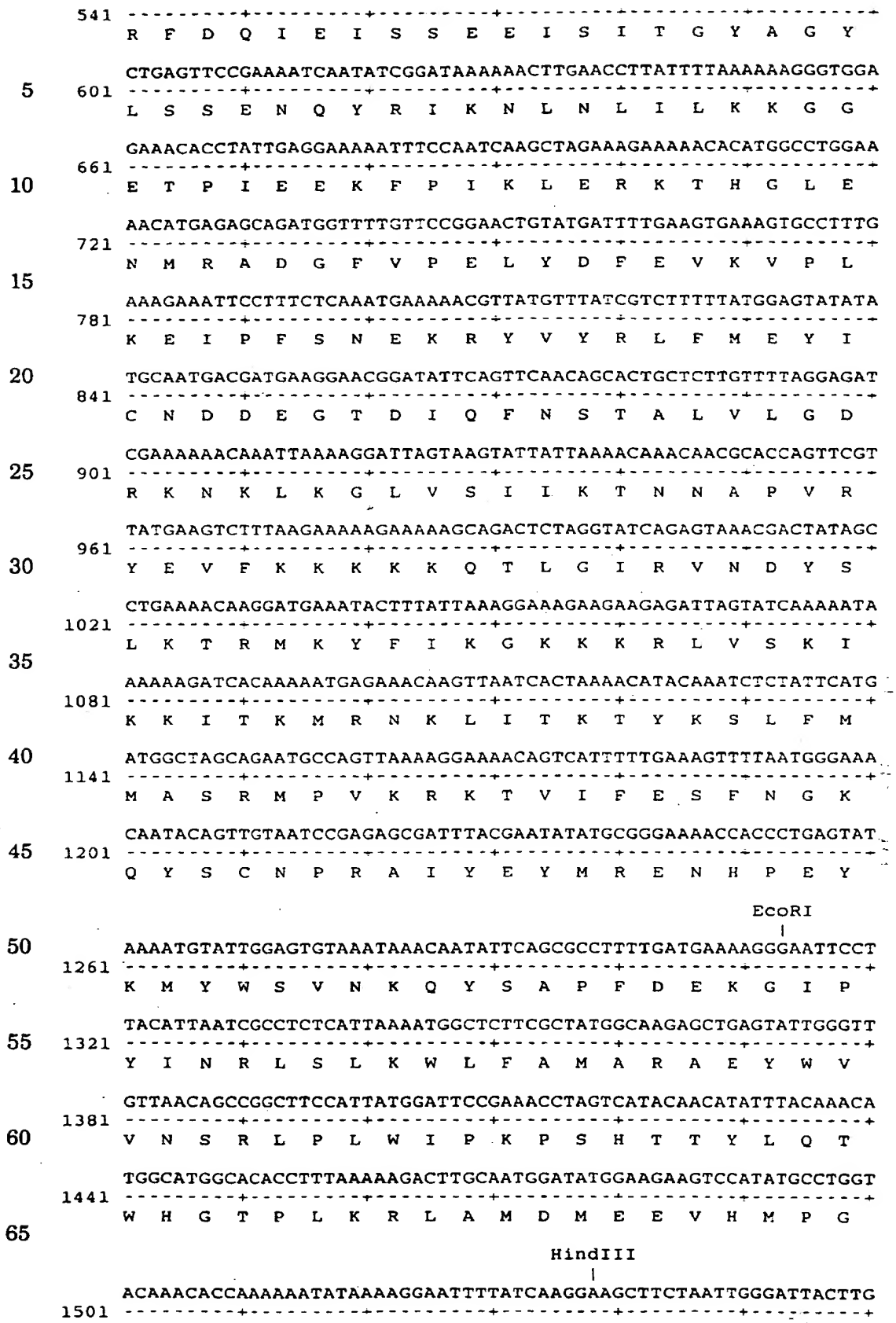
          GATTCAGAACACCAATTTTTAGAGGATTACCAGTTATTTTTAAAGCTGAAAGAGAGACGT
       181 ---------+---------+---------+---------+---------+---------+
40        D  S  E  H  Q  F  L  E  D  Y  Q  L  F  L  K  L  K  E  R  R

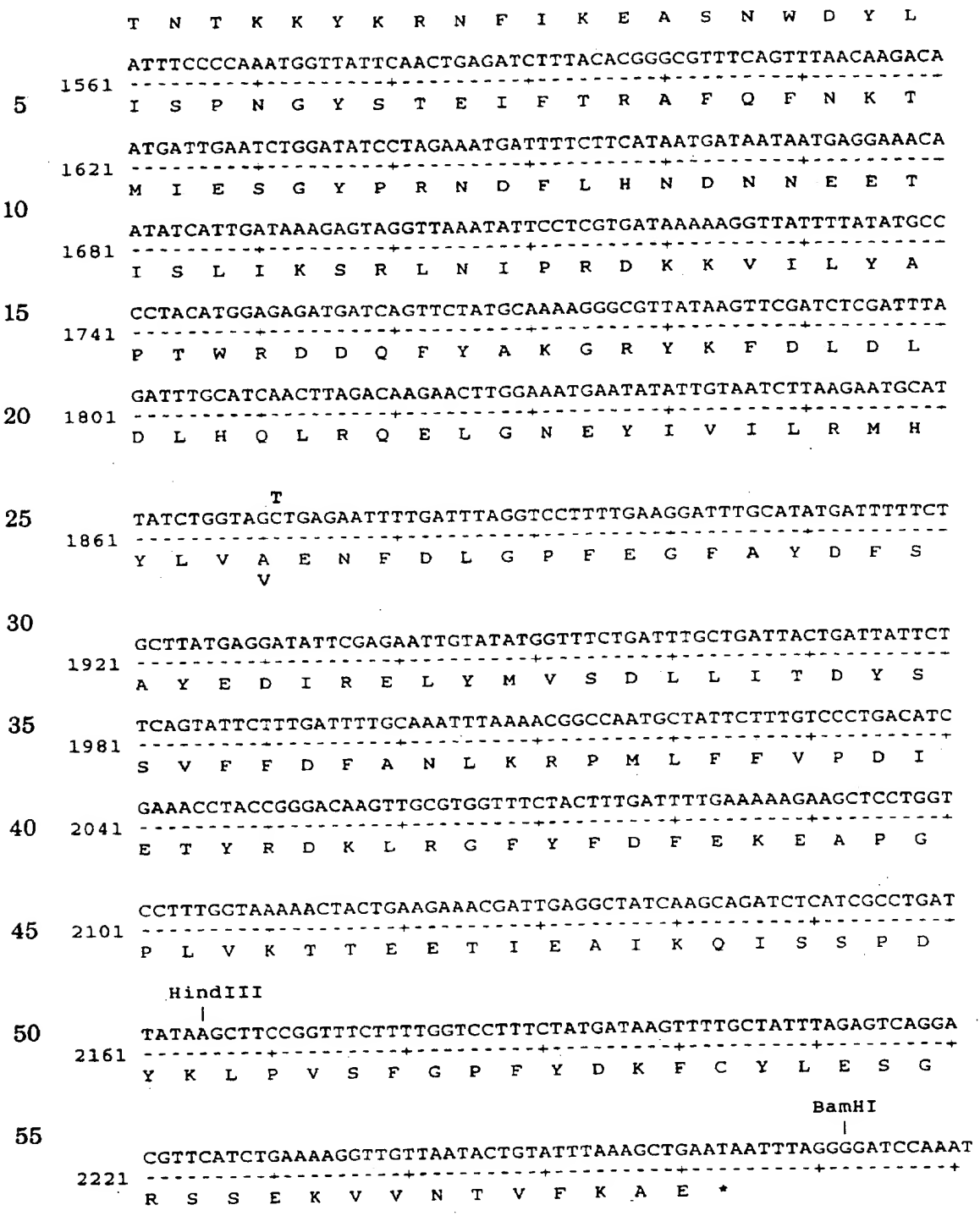
          TCAGGAACGGAATCTGAATTTCCGCTTCAAAACACTGGCTCATTAGAGTATAAAACTGAG
       241 ---------+---------+---------+---------+---------+---------+
          S  G  T  E  S  E  F  P  L  Q  N  T  G  S  L  E  Y  K  T  E
45
          ATAAATGCTCATGTTTTGCCTATGCCTGTTGAAATGGGACAAACATATGATTTTTATGTC
       301 ---------+---------+---------+---------+---------+---------+
          I  N  A  H  V  L  P  M  P  V  E  M  G  Q  T  Y  D  F  Y  V

50        GAATTTCGAAAAAAATATGAAGATGCGGAGCAGGAACCACTCTTGAAGCGTCTTTCTGCT
       361 ---------+---------+---------+---------+---------+---------+
          E  F  R  K  K  Y  E  D  A  E  Q  E  P  L  L  K  R  L  S  A

          GAAGTAAATTCAATTGAGCGCGCCTTTCATGTCGATCAAACCACAGAACTTTTGATTTTA
55     421 ---------+---------+---------+---------+---------+---------+
          E  V  N  S  I  E  R  A  F  H  V  D  Q  T  T  E  L  L  I  L

          CCTTATACAACTGATAAAGGCAACTTTTCTATTAAGGTGAAAAGAGAGGCCAAAATCATC
       481 ---------+---------+---------+---------+---------+---------+
60        P  Y  T  T  D  K  G  N  F  S  I  K  V  K  R  E  A  K  I  I
                                                                  KpnI
                                                                   |
          AGATTTGATCAAATCGAGATTAGCTCTGAAGAAATAAGCATAACAGGTTATGCGGGGTAC
```





SEQUENCE CHART 5

The putative amino acids, or protein from SEQUENCE CHART 4, including the single amino acid mutation, shown below. The amino acids from this Sequence Chart 5 are listed separately as SEQ. ID. NO. 6 (SEQ. ID. NO. 6 is the sequence without the mutation at position 616).

1	M	S	L	V	V	D	T	N	K	R	K	Q							
13	K	G	K	S	F	Y	T	E	E	Q	K	K	V	M	I	E	N	T	V	I
33	K	C	I	L	K	S	L	K	N	N	L	G	S	L	E	L	L	I	S	I
53	D	S	E	H	Q	F	L	E	D	Y	Q	L	F	L	K	L	K	E	R	R
73	S	G	T	E	S	E	F	P	L	Q	N	T	G	S	L	E	Y	K	T	E
93	I	N	A	H	V	L	P	M	P	V	E	M	G	Q	T	Y	D	F	Y	V
113	E	F	R	K	K	Y	E	D	A	E	Q	E	P	L	L	K	R	L	S	A
133	E	V	N	S	I	E	R	A	F	H	V	D	Q	T	T	E	L	L	I	L
153	P	Y	T	T	D	K	G	N	F	S	I	K	V	K	R	E	A	K	I	I
173	R	F	D	Q	I	E	I	S	S	E	E	I	S	I	T	G	Y	A	G	Y
193	L	S	S	E	N	Q	Y	R	I	K	N	L	N	L	I	L	K	K	G	G
213	E	T	P	I	E	E	K	F	P	I	K	L	E	R	K	T	H	G	L	E
233	N	M	R	A	D	G	F	V	P	E	L	Y	D	F	E	V	K	V	P	L
253	K	E	I	P	F	S	N	E	K	R	Y	V	Y	R	L	F	M	E	Y	I
273	C	N	D	D	E	G	T	D	I	Q	F	N	S	T	A	L	V	L	G	D
293	R	K	N	K	L	K	G	L	V	S	I	I	K	T	N	N	A	P	V	R
313	Y	E	V	F	K	K	K	K	K	Q	T	L	G	I	R	V	N	D	Y	S
333	L	K	T	R	M	K	Y	F	I	K	G	K	K	K	R	L	V	S	K	I
353	K	K	I	T	K	M	R	N	K	L	I	T	K	T	Y	K	S	L	F	M
373	M	A	S	R	M	P	V	K	R	K	T	V	I	F	E	S	F	N	G	K
393	Q	Y	S	C	N	P	R	A	I	Y	E	Y	M	R	E	N	H	P	E	Y
413	K	M	Y	W	S	V	N	K	Q	Y	S	A	P	F	D	E	K	G	I	P
433	Y	I	N	R	L	S	L	K	W	L	F	A	M	A	R	A	E	Y	W	V
453	V	N	S	R	L	P	L	W	I	P	K	P	S	H	T	T	Y	L	Q	T
473	W	H	G	T	P	L	K	R	L	A	M	D	M	E	E	V	H	M	P	G
493	T	N	T	K	K	Y	K	R	N	F	I	K	E	A	S	N	W	D	Y	L
513	I	S	P	N	G	Y	S	T	E	I	F	T	R	A	F	Q	F	N	K	T
533	M	I	E	S	G	Y	P	R	N	D	F	L	H	N	D	N	N	E	E	T
553	I	S	L	I	K	S	R	L	N	I	P	R	D	K	K	V	I	L	Y	A
573	P	T	W	R	D	D	Q	F	Y	A	K	G	R	Y	K	F	D	L	D	L

593	D	L	H	Q	L	R	Q	E	L	G	N	E	Y	I	V	I	L	R	M	H
613	Y	L	V	A V	E	N	F	D	L	G	P	F	E	G	F	A	Y	D	F	S
633	A	Y	E	D	I	R	E	L	Y	M	V	S	D	L	L	I	T	D	Y	S
653	S	V	F	F	D	F	A	N	L	K	R	P	M	L	F	F	V	P	D	I
673	E	T	Y	R	D	K	L	R	G	F	Y	F	D	F	E	K	E	A	P	G
693	P	L	V	K	T	T	E	E	T	I	E	A	I	K	Q	I	S	S	P	D
713	Y	K	L	P	V	S	F	G	P	F	Y	D	K	F	C	Y	L	E	S	G
733	R	S	S	E	K	V	V	N	T	V	F	K	A	E	-	746				

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Pharmacia and Upjohn Co.

(ii) TITLE OF INVENTION: TEICHOIC ACID ENZYMES AND ASSAYS

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pharmacia and Upjohn, Co., Intel. Prop. Law (1920-32-LAW)
(B) STREET: 301 Henrietta Street
(C) CITY: Kalamazoo
(D) STATE: Michigan
(E) COUNTRY: U.S.A.
(F) ZIP: 49001

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Wootton, Thomas A.
(B) REGISTRATION NUMBER: 35,004
(C) REFERENCE/DOCKET NUMBER: 6084

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (616) 833-7914
(B) TELEFAX: (616) 833-8897

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2182 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGATTGAAA ACACTGTGAT TAAATGTATT TTGAAAAGCT TGAAAAACAA TTTAGGAAGT 60

CTTGAATTGT TAATCTCAAT TGATTCAGAA CACCAATTTT TAGAGGATTA CCAGTTATTT 120

TTAAAGCTGA AAGAGAGACG TTCAGGAACG GAATCTGAAT TTCCGCTTCA AAACACTGGC 180

TCATTAGAGT ATAAAACTGA GATAAATGCT CATGTTTTGC CTATGCCTGT TGAAATGGGA 240

CAAACATATG ATTTTTATGT CGAATTTCGA AAAAAATATG AAGATGCGGA GCAGGAACCA 300

CTCTTGAAGC GTCTTTCTGC TGAAGTAAAT TCAATTGAGC GCGCCTTTCA TGTCGATCAA 360

ACCACAGAAC TTTTGATTTT ACCTTATACA ACTGATAAAG GCAACTTTTC TATTAAGGTG 420

AAAAGAGAGG CCAAAATCAT CAGATTTGAT CAAATCGAGA TTAGCTCTGA AGAAATAAGC 480

ATAACAGGTT ATGCGGGGTA CCTGAGTTCC GAAAATCAAT ATCGGATAAA AAACTTGAAC 540

CTTATTTTAA AAAAGGGTGG AGAAACACCT ATTGAGGAAA AATTTCCAAT CAAGCTAGAA 600

AGAAAAACAC ATGGCCTGGA AAACATGAGA GCAGATGGTT TTGTTCCGGA ACTGTATGAT 660

TTTGAAGTGA AAGTGCCTTT GAAAGAAATT CCTTTCTCAA ATGAAAAACG TTATGTTTAT 720

CGTCTTTTTA TGGAGTATAT ATGCAATGAC GATGAAGGAA CGGATATTCA GTTCAACAGC 780

ACTGCTCTTG TTTTAGGAGA TCGAAAAAAC AAATTAAAAG GATTAGTAAG TATTATTAAA 840

ACAAACAACG CACCAGTTCG TTATGAAGTC TTTAAGAAAA AGAAAAAGCA GACTCTAGGT 900

ATCAGAGTAA ACGACTATAG CCTGAAAACA AGGATGAAAT ACTTTATTAA AGGAAAGAAG 960

AAGAGATTAG TATCAAAAAT AAAAAAGATC ACAAAAATGA GAAACAAGTT AATCACTAAA 1020

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TTTGAAAGTT TTAATGGGAA ACAATACAGT TCTAATCCGA GAGCGATTTA CGAATATATG 1140

CGGGAAAACC ACCCTGAGTA TAAAATGTAT TGGAGTGTAA ATAAACAATA TTCAGCGCCT 1200

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GAAGAAGTCC ATATGCCTGG TACAAACACC AAAAAATATA AAAGGAATTT TATCAAGGAA 1440

GCTTCTAATT GGGATTACTT GATTTCCCCA AATGGTTATT CAACTGAGAT CTTTACACGG 1500

GCGTTTCAGT TTAACAAGAC AATGATTGAA TCTGGATATC CTAGAAATGA TTTTCTTCAT 1560

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AAAAAGGTTA TTTTATATGC CCCTACATGG AGAGATGATC AGTTCTATGC AAAAGGGCGT 1680

TATAAGTTCG ATCTCGATTT AGATTTGCAT CAACTTAGAC AAGAACTTGG AAATGAATAT 1740

ATTGTAATCT TAAGAATGCA TTATCTGGTA GCTGAGAATT TTGATTTAGG TCCTTTTGAA 1800

GGATTTGCAT ATGATTTTTC TGCTTATGAG GATATTCGAG AATTGTATAT GGTTTCTGAT 1860

TTGCTGATTA CTGATTATTC TTCAGTATTC TTTGATTTTG CAAATTTAAA ACGGCCAATG 1920

CTATTCTTTG TCCCTGACAT CGAAACCTAC CGGGACAAGT TGCGTGGTTT CTACTTTGAT 1980

TTTGAAAAAG AAGCTCCTGG TCCTTTGGTA AAAACTACTG AAGAAACGAT TGAGGCTATC 2040

AAGCAGATCT CATCGCCTGA TTATAAGCTT CCGGTTTCTT TTGGTCCTTT CTATGATAAG 2100

TTTTGCTATT TAGAGTCAGG ACGTTCATCT GAAAAGGTTG TTAATACTGT ATTTAAAGCT 2160

GAATAATTTA GGGGATCCAA AT 2182

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 721 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1               5                  10                  15

Asn Leu Gly Ser Leu Glu Leu Leu Ile Ser Ile Asp Ser Glu His Gln
            20                  25                  30

Phe Leu Glu Asp Tyr Gln Leu Phe Leu Lys Leu Lys Glu Arg Arg Ser
        35                  40                  45

Gly Thr Glu Ser Glu Phe Pro Leu Gln Asn Thr Gly Ser Leu Glu Tyr
    50                  55                  60

Lys Thr Glu Ile Asn Ala His Val Leu Pro Met Pro Val Glu Met Gly
65                  70                  75                  80

Gln Thr Tyr Asp Phe Tyr Val Glu Phe Arg Lys Lys Tyr Glu Asp Ala
                85                  90                  95

Glu Gln Glu Pro Leu Leu Lys Arg Leu Ser Ala Glu Val Asn Ser Ile
            100                 105                 110

Glu Arg Ala Phe His Val Asp Gln Thr Thr Glu Leu Leu Ile Leu Pro
        115                 120                 125

Tyr Thr Thr Asp Lys Gly Asn Phe Ser Ile Lys Val Lys Arg Glu Ala
    130                 135                 140

Lys Ile Ile Arg Phe Asp Gln Ile Glu Ile Ser Ser Glu Glu Ile Ser
145                 150                 155                 160

Ile Thr Gly Tyr Ala Gly Tyr Leu Ser Ser Glu Asn Gln Tyr Arg Ile
                165                 170                 175

Lys Asn Leu Asn Leu Ile Leu Lys Lys Gly Gly Glu Thr Pro Ile Glu
            180                 185                 190

Glu Lys Phe Pro Ile Lys Leu Glu Arg Lys Thr His Gly Leu Glu Asn
        195                 200                 205

Met Arg Ala Asp Gly Phe Val Pro Glu Leu Tyr Asp Phe Glu Val Lys
    210                 215                 220

Val Pro Leu Lys Glu Ile Pro Phe Ser Asn Glu Lys Arg Tyr Val Tyr
225                 230                 235                 240

Arg Leu Phe Met Glu Tyr Ile Cys Asn Asp Asp Glu Gly Thr Asp Ile
                245                 250                 255

Gln Phe Asn Ser Thr Ala Leu Val Leu Gly Asp Arg Lys Asn Lys Leu
            260                 265                 270

Lys Gly Leu Val Ser Ile Ile Lys Thr Asn Asn Ala Pro Val Arg Tyr
        275                 280                 285
```

Glu Val Phe Lys Lys Lys Lys Lys Gln Thr Leu Gly Ile Arg Val Asn
290 295 300

Asp Tyr Ser Leu Lys Thr Arg Met Lys Tyr Phe Ile Lys Gly Lys Lys
305 310 315 320

Lys Arg Leu Val Ser Lys Ile Lys Lys Ile Thr Lys Met Arg Asn Lys
325 330 335

Leu Ile Thr Lys Thr Tyr Lys Ser Leu Phe Met Met Ala Ser Arg Met
340 345 350

Pro Val Lys Arg Lys Thr Val Ile Phe Glu Ser Phe Asn Gly Lys Gln
355 360 365

Tyr Ser Cys Asn Pro Arg Ala Ile Tyr Glu Tyr Met Arg Glu Asn His
370 375 380

Pro Glu Tyr Lys Met Tyr Trp Ser Val Asn Lys Gln Tyr Ser Ala Pro
385 390 395 400

Phe Asp Glu Lys Gly Ile Pro Tyr Ile Asn Arg Leu Ser Leu Lys Trp
405 410 415

Leu Phe Ala Met Ala Arg Ala Glu Tyr Trp Val Val Asn Ser Arg Leu
420 425 430

Pro Leu Trp Ile Pro Lys Pro Ser His Thr Thr Tyr Leu Gln Thr Trp
435 440 445

His Gly Thr Pro Leu Lys Arg Leu Ala Met Asp Met Glu Glu Val His
450 455 460

Met Pro Gly Thr Asn Thr Lys Lys Tyr Lys Arg Asn Phe Ile Lys Glu
465 470 475 480

Ala Ser Asn Trp Asp Tyr Leu Ile Ser Pro Asn Gly Tyr Ser Thr Glu
485 490 495

Ile Phe Thr Arg Ala Phe Gln Phe Asn Lys Thr Met Ile Glu Ser Gly
500 505 510

Tyr Pro Arg Asn Asp Phe Leu His Asn Asp Asn Asn Glu Glu Thr Ile
515 520 525

Ser Leu Ile Lys Ser Arg Leu Asn Ile Pro Arg Asp Lys Lys Val Ile
530 535 540

Leu Tyr Ala Pro Thr Trp Arg Asp Asp Gln Phe Tyr Ala Lys Gly Arg
545 550 555 560

Tyr Lys Phe Asp Leu Asp Leu Asp Leu His Gln Leu Arg Gln Glu Leu
565 570 575

Gly Asn Glu Tyr Ile Val Ile Leu Arg Met His Tyr Leu Val Ala Glu
580 585 590

Asn Phe Asp Leu Gly Pro Phe Glu Gly Phe Ala Tyr Asp Phe Ser Ala
595 600 605

Tyr Glu Asp Ile Arg Glu Leu Tyr Met Val Ser Asp Leu Leu Ile Thr
610 615 620

Asp Tyr Ser Ser Val Phe Phe Asp Phe Ala Asn Leu Lys Arg Pro Met
625 630 635 640

Leu Phe Phe Val Pro Asp Ile Glu Thr Tyr Arg Asp Lys Leu Arg Gly
645 650 655

Phe Tyr Phe Asp Phe Glu Lys Glu Ala Pro Gly Pro Leu Val Lys Thr
660 665 670

Thr Glu Glu Thr Ile Glu Ala Ile Lys Gln Ile Ser Ser Pro Asp Tyr
675 680 685

Lys Leu Pro Val Ser Phe Gly Pro Phe Tyr Asp Lys Phe Cys Tyr Leu
690 695 700

Glu Ser Gly Arg Ser Ser Glu Lys Val Val Asn Thr Val Phe Lys Ala
705 710 715 720

Glu

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTCAGGATCC TTCTCTTGGA GGGTCACGGA AATAAAAG 38

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATTTGGATCC CCTAAATTAT TCAGCTTTAA ATAC 34

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2281 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTTGGATCC AAGGAAGAGA GTTAATGTCC TTAGTAGTTG ACACTAATAA AAGGAAGCAA 60
AAAGGAAAGA GCTTTTATAC AGAGGAGCAG AAAAAAGTAA TGATTGAAAA CACTGTGATT 120
AAATGTATTT TGAAAAGCTT GAAAAACAAT TTAGGAAGTC TTGAATTGTT AATCTCAATT 180
GATTCAGAAC ACCAATTTTT AGAGGATTAC CAGTTATTTT TAAAGCTGAA AGAGAGACGT 240
TCAGGAACGG AATCTGAATT TCCGCTTCAA AACACTGGCT CATTAGAGTA TAAAACTGAG 300
ATAAATGCTC ATGTTTTGCC TATGCCTGTT GAAATGGGAC AAACATATGA TTTTTATGTC 360
GAATTTCGAA AAAAATATGA AGATGCGGAG CAGGAACCAC TCTTGAAGCG TCTTTCTGCT 420
GAAGTAAATT CAATTGAGCG CGCCTTTCAT GTCGATCAAA CCACAGAACT TTTGATTTTA 480
CCTTATACAA CTGATAAAGG CAACTTTTCT ATTAAGGTGA AAAGAGAGGC CAAAATCATC 540
AGATTTGATC AAATCGAGAT TAGCTCTGAA GAAATAAGCA TAACAGGTTA TGCGGGGTAC 600
CTGAGTTCCG AAAATCAATA TCGGATAAAA AACTTGAACC TTATTTTAAA AAAGGGTGGA 660
GAAACACCTA TTGAGGAAAA ATTTCCAATC AAGCTAGAAA GAAAAACACA TGGCCTGGAA 720
AACATGAGAG CAGATGGTTT TGTTCCGGAA CTGTATGATT TTGAAGTGAA AGTGCCTTTG 780
AAAGAAATTC CTTTCTCAAA TGAAAAACGT TATGTTTATC GTCTTTTTAT GGAGTATATA 840
TGCAATGACG ATGAAGGAAC GGATATTCAG TTCAACAGCA CTGCTCTTGT TTTAGGAGAT 900
CGAAAAAACA AATTAAAAGG ATTAGTAAGT ATTATTAAAA CAAACAACGC ACCAGTTCGT 960
TATGAAGTCT TTAAGAAAAA GAAAAAGCAG ACTCTAGGTA TCAGAGTAAA CGACTATAGC 1020
CTGAAAACAA GGATGAAATA CTTTATTAAA GGAAAGAAGA AGAGATTAGT ATCAAAAATA 1080
AAAAAGATCA CAAAAATGAG AAACAAGTTA ATCACTAAAA CATACAAATC TCTATTCATG 1140
ATGGCTAGCA GAATGCCAGT TAAAAGGAAA ACAGTCATTT TTGAAAGTTT TAATGGGAAA 1200
CAATACAGTT GTAATCCGAG AGCGATTTAC GAATATATGC GGGAAAACCA CCCTGAGTAT 1260
AAAATGTATT GGAGTGTAAA TAAACAATAT TCAGCGCCTT TTGATGAAAA GGGAATTCCT 1320
TACATTAATC GCCTCTCATT AAAATGGCTC TTCGCTATGG CAAGAGCTGA GTATTGGGTT 1380
GTTAACAGCC GGCTTCCATT ATGGATTCCG AAACCTAGTC ATACAACATA TTTACAAACA 1440
TGGCATGGCA CACCTTTAAA AAGACTTGCA ATGGATATGG AAGAAGTCCA TATGCCTGGT 1500
ACAAACACCA AAAAATATAA AAGGAATTTT ATCAAGGAAG CTTCTAATTG GGATTACTTG 1560
ATTTCCCCAA ATGGTTATTC AACTGAGATC TTTACACGGG CGTTTCAGTT TAACAAGACA 1620
ATGATTGAAT CTGGATATCC TAGAAATGAT TTTCTTCATA ATGATAATAA TGAGGAAACA 1680
ATATCATTGA TAAAGAGTAG GTTAAATATT CCTCGTGATA AAAAGGTTAT TTTATATGCC 1740
CCTACATGGA GAGATGATCA GTTCTATGCA AAAGGGCGTT ATAAGTTCGA TCTCGATTTA 1800
GATTTGCATC AACTTAGACA AGAACTTGGA AATGAATATA TTGTAATCTT AAGAATGCAT 1860
TATCTGGTAG CTGAGAATTT TGATTTAGGT CCTTTTGAAG GATTTGCATA TGATTTTTCT 1920
GCTTATGAGG ATATTCGAGA ATTGTATATG GTTTCTGATT TGCTGATTAC TGATTATTCT 1980

TCAGTATTCT TTGATTTTGC AAATTTAAAA CGGCCAATGC TATTCTTTGT CCCTGACATC 2040

GAAACCTACC GGGACAAGTT GCGTGGTTTC TACTTTGATT TTGAAAAAGA AGCTCCTGGT 2100

CCTTTGGTAA AAACTACTGA AGAAACGATT GAGGCTATCA AGCAGATCTC ATCGCCTGAT 2160

TATAAGCTTC CGGTTTCTTT TGGTCCTTTC TATGATAAGT TTTGCTATTT AGAGTCAGGA 2220

CGTTCATCTG AAAAGGTTGT TAATACTGTA TTTAAAGCTG AATAATTTAG GGGATCCAAA 2280

T 2281

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 746 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ser Leu Val Val Asp Thr Asn Lys Arg Lys Gln Lys Gly Lys Ser
1 5 10 15

Phe Tyr Thr Glu Glu Gln Lys Lys Val Met Ile Glu Asn Thr Val Ile
20 25 30

Lys Cys Ile Leu Lys Ser Leu Lys Asn Asn Leu Gly Ser Leu Glu Leu
35 40 45

Leu Ile Ser Ile Asp Ser Glu His Gln Phe Leu Glu Asp Tyr Gln Leu
50 55 60

Phe Leu Lys Leu Lys Glu Arg Arg Ser Gly Thr Glu Ser Glu Phe Pro
65 70 75 80

Leu Gln Asn Thr Gly Ser Leu Glu Tyr Lys Thr Glu Ile Asn Ala His
85 90 95

Val Leu Pro Met Pro Val Glu Met Gly Gln Thr Tyr Asp Phe Tyr Val
100 105 110

Glu Phe Arg Lys Lys Tyr Glu Asp Ala Glu Gln Glu Pro Leu Leu Lys
115 120 125

Arg Leu Ser Ala Glu Val Asn Ser Ile Glu Arg Ala Phe His Val Asp
130 135 140

Gln Thr Thr Glu Leu Leu Ile Leu Pro Tyr Thr Thr Asp Lys Gly Asn
145 150 155 160

Phe Ser Ile Lys Val Lys Arg Glu Ala Lys Ile Ile Arg Phe Asp Gln
165 170 175

Ile Glu Ile Ser Ser Glu Glu Ile Ser Ile Thr Gly Tyr Ala Gly Tyr
180 185 190

Leu Ser Ser Glu Asn Gln Tyr Arg Ile Lys Asn Leu Asn Leu Ile Leu

195 200 205

Lys Lys Gly Gly Glu Thr Pro Ile Glu Glu Lys Phe Pro Ile Lys Leu
210 215 220

Glu Arg Lys Thr His Gly Leu Glu Asn Met Arg Ala Asp Gly Phe Val
225 230 235 240

Pro Glu Leu Tyr Asp Phe Glu Val Lys Val Pro Leu Lys Glu Ile Pro
245 250 255

Phe Ser Asn Glu Lys Arg Tyr Val Tyr Arg Leu Phe Met Glu Tyr Ile
260 265 270

Cys Asn Asp Asp Glu Gly Thr Asp Ile Gln Phe Asn Ser Thr Ala Leu
275 280 285

Val Leu Gly Asp Arg Lys Asn Lys Leu Lys Gly Leu Val Ser Ile Ile
290 295 300

Lys Thr Asn Asn Ala Pro Val Arg Tyr Glu Val Phe Lys Lys Lys Lys
305 310 315 320

Lys Gln Thr Leu Gly Ile Arg Val Asn Asp Tyr Ser Leu Lys Thr Arg
325 330 335

Met Lys Tyr Phe Ile Lys Gly Lys Lys Lys Arg Leu Val Ser Lys Ile
340 345 350

Lys Lys Ile Thr Lys Met Arg Asn Lys Leu Ile Thr Lys Thr Tyr Lys
355 360 365

Ser Leu Phe Met Met Ala Ser Arg Met Pro Val Lys Arg Lys Thr Val
370 375 380

Ile Phe Glu Ser Phe Asn Gly Lys Gln Tyr Ser Cys Asn Pro Arg Ala
385 390 395 400

Ile Tyr Glu Tyr Met Arg Glu Asn His Pro Glu Tyr Lys Met Tyr Trp
405 410 415

Ser Val Asn Lys Gln Tyr Ser Ala Pro Phe Asp Glu Lys Gly Ile Pro
420 425 430

Tyr Ile Asn Arg Leu Ser Leu Lys Trp Leu Phe Ala Met Ala Arg Ala
435 440 445

Glu Tyr Trp Val Val Asn Ser Arg Leu Pro Leu Trp Ile Pro Lys Pro
450 455 460

Ser His Thr Thr Tyr Leu Gln Thr Trp His Gly Thr Pro Leu Lys Arg
465 470 475 480

Leu Ala Met Asp Met Glu Glu Val His Met Pro Gly Thr Asn Thr Lys
485 490 495

Lys Tyr Lys Arg Asn Phe Ile Lys Glu Ala Ser Asn Trp Asp Tyr Leu
500 505 510

Ile Ser Pro Asn Gly Tyr Ser Thr Glu Ile Phe Thr Arg Ala Phe Gln
515 520 525

Phe Asn Lys Thr Met Ile Glu Ser Gly Tyr Pro Arg Asn Asp Phe Leu
530 535 540

His Asn Asp Asn Asn Glu Glu Thr Ile Ser Leu Ile Lys Ser Arg Leu
545 550 555 560

Asn Ile Pro Arg Asp Lys Lys Val Ile Leu Tyr Ala Pro Thr Trp Arg

565 570 575

Asp Asp Gln Phe Tyr Ala Lys Gly Arg Tyr Lys Phe Asp Leu Asp Leu
580 585 590

Asp Leu His Gln Leu Arg Gln Glu Leu Gly Asn Glu Tyr Ile Val Ile
595 600 605

Leu Arg Met His Tyr Leu Val Ala Glu Asn Phe Asp Leu Gly Pro Phe
610 615 620

Glu Gly Phe Ala Tyr Asp Phe Ser Ala Tyr Glu Asp Ile Arg Glu Leu
625 630 635 640

Tyr Met Val Ser Asp Leu Leu Ile Thr Asp Tyr Ser Ser Val Phe Phe
645 650 655

Asp Phe Ala Asn Leu Lys Arg Pro Met Leu Phe Phe Val Pro Asp Ile
660 665 670

Glu Thr Tyr Arg Asp Lys Leu Arg Gly Phe Tyr Phe Asp Phe Glu Lys
675 680 685

Glu Ala Pro Gly Pro Leu Val Lys Thr Thr Glu Glu Thr Ile Glu Ala
690 695 700

Ile Lys Gln Ile Ser Ser Pro Asp Tyr Lys Leu Pro Val Ser Phe Gly
705 710 715 720

Pro Phe Tyr Asp Lys Phe Cys Tyr Leu Glu Ser Gly Arg Ser Ser Glu
725 730 735

Lys Val Val Asn Thr Val Phe Lys Ala Glu
740 745

CLAIMS

(SUBSTRATE and ASSAY)

1*. The use of lipoteichoic acid as a substrate for the enzymatic reaction catalyzed by the TAP protein.

2. The use of lipoteichoic acid as in claim 1, where the lipoteichoic acid is isolated or purified from *B. subtilis*, *S. aureus*, or *E. faecalis*, or obtained from a commercially available source.

3. The use of lipoteichoic acid as in claim 1, where the lipoteichoic acid is prepared from *B. subtilis*, *S. aureus*, or *E. faecalis*.

4. The use of lipoteichoic acid as in claim 3, where the lipoteichoic acid serves as an acceptor of CDP[^{3}H]glycerol.

5. The use of lipoteichoic acid as in claim 1, where the TAP protein is a peptide or protein expressed from a cloned cell where the peptide or protein comprises a peptide or protein that is at least about 70% homologous to the amino acid residues of Chart 2, or SEQ. ID. No. 2.

6. The use of lipoteichoic acid as in claim 5, where the TAP protein is a peptide or protein comprised of amino acid residues substantially the same as the amino acid residues of Chart 2, or SEQ. ID. No. 2.

7. A method of measuring the activity of TAP enzyme comprising, combining CDP-glycerol (which contains glycerol-3-phosphate), plus H_2O, plus TAP enzyme, plus lipoteichoic acid, and measuring the amount of glycerol-3-phosphate that is transferred to lipoteichoic acid.

8. The method of claim 7 where the CDP-glycerol is radioactive CDP-glycerol.

9. The method of claim 8 where the radioactive CDP-glycerol is made from [^{3}H]glycerol-3-phosphate (a.k.a. [^{3}H]glycerophosphate).

10. The method claim 7, where the lipoteichoic acid is treated to remove alanine before it is combined with the other ingredients.

11. The method of claim 8, wh re radioactive CDP-glycerol , plus H_2O, plus TAP enzyme, plus lipoteichoic acid, plus strepavidin SPA beads and a suitable lectin such as a wheat germ agglutinin are combined and the amount of glycerol-3-phosphate that is transferred to lipoteichoic acid is measured by measuring the lectin bound to the SPA beads.

12. The method of claims 7, where the TAP enzyme is from any impure preparation.

13. The method of claim 7, where the TAP enzyme is a peptide or protein expressed from a cloned cell where the expressed protein comprises a peptide or protein that has at least about 90% identity to the first 20 N-Terminal amino residues of SEQ. ID. NO. 2 and is at least about 70% homologous to the entire amino acid residues of Chart 2, or SEQ. ID. No. 2.

14. The method of claim 7, where the TAP enzyme is substantially the same as the protein disclosed in SEQ. ID. NO. 2.

(PROTEIN)

15*. A peptide or protein expressed from a cloned cell where the peptide or protein comprises a peptide or protein that has at least about 90% homology to the first 20 N-Terminal amino residues of SEQ. ID. NO. 2 and is at least about 70% homologous to the entire amino acid residues of Chart 2, or SEQ. ID. No. 2.

16. A peptide or protein of claim 15 being at least about 80% homologous to the entire protein in Chart 2, or SEQ. ID. No. 2.

17. A peptide or protein of claim 16 being at least about 90% homologous to the entire protein in Chart 2, or SEQ. ID. No. 2.

18. A peptide or protein of claim 17 being at least about 95% homologous to the entire protein in Chart 2, or SEQ. ID. No. 2.

19. A peptide or protein of claim 18 comprising residues substantially the same or similar to those in Chart 2, or SEQ. ID. No. 2.

20. A peptide or protein of claim 19 comprising the residues disclosed in Chart 2, or SEQ. ID. No. 2.

21. A peptide or protein of claim 19 where valine is the amino acid at position 616 in place of alanine.

(DNA)

22*. A cloned nucleic acid residue sequence comprising a nucleic acid residue sequence having the ability to catalyze the reaction of CDP-glycerol plus H_2O into teichoic or lipoteichoic acid, having at least about 90 % homology to the first 20 nucleic acid residues of SEQ. ID. NO. 1 (5' end) and capable of hybridizing to the DNA sequence of SEQ. ID. NO. 1, under standard stringent conditions to about 70 or more percent homology.

23. A cloned nucleic acid residue sequence of claim 22, capable of hybridizing to the DNA sequence of SEQ. ID. NO. 1, under standard stringent conditions to about 80 or more percent homology.

24. A cloned nucleic acid residue sequence of claim 23, capable of hybridizing to the DNA sequence of SEQ. ID. NO. 1, under standard stringent conditions to about 90 or more percent homology.

25. A cloned nucleic acid residue sequence of claim 24, capable of hybridizing to the DNA sequence of SEQ. ID. NO. 1, under standard stringent conditions to about 95 or more percent homology.

26. A cloned nucleic acid residue sequence of claim 25, comprising residues substantially the same or similar as those in the DNA sequence of SEQ. ID. NO. 1.

27. A cloned nucleic acid residue sequence of claim 26, comprising the residues shown in the DNA sequence of SEQ. ID. NO. 1.

28. A fragment of the nucleic acid residue sequence of claim 27, comprising the residues from residue 4 to 2274, or from the first to the last restriction site, as indicated on Chart 2.

29. A fragment of the nucleic acid residue sequence of claim 27, comprising the residues from residue 24 to 2264, or from the first start to the last stop codons, as indicated in Chart 2.

30. The nucleic acids of claim 26 comprising the nucleic acids that code for the protein described in SEQ. ID. NO. 2.

31. A cloned nucleic acid residue sequence of claim 26, where the residue at position 1872 is tyrosine in place of cystine, where the protein expressed from this sequence expresses a valine in place of alanine at position 616 of the expressed peptide.

32*. An isolated nucleic acid residue sequence fragment from *Staphylococcus aureus* where said sequence fragment is about 7.0, 5.0 or 4.2 K bases, where said sequence fragment is produced from EcoR1 digest of *Staphylococcus aureus* genome, where the sequence of said sequence fragment is at least about 70% homologous to related residues in SEQ. ID. No. 2.

33*. An isolated nucleic acid residue sequence fragment from *Staphylococcus aureus* where the sequence fragment is about 4.5, 3.3 or 2.8 K bases, where said sequence fragment is produced from a HindIII digest of *Staphylococcus aureus* genome, where said sequence fragment is at least about 70% homologous to residues in SEQ. ID. No. 2.

(VECTORS)

34*. A vector, comprising either a cloning vector, a shuttle vector or an expression vector, having a cloned nucleic acid residue sequence comprising a nucleic acid residue sequence having the ability to catalyze the reaction of CDP-glycerol plus H_2O into teichoic or lipoteichoic acid, having 90% homology to the first 20 nucleic acid residues of SEQ. ID. NO. 1 (5' end) and capable of hybridizing to the DNA sequence of SEQ. ID. NO. 1, under standard stringent conditions to about 70 or

more percent homology,

35. A plasmid of claim 34, comprised of a plasmid selected from any plasmid suitable as a cloning vector.

36. A plasmid of claim 35, selected from any suitable, widely available or commercially available plasmids.

37. A plasmid of claim 36 selected from any suitable pUC or suitable pBR plasmid.

38. A plasmid of claim 37, selected from pUC18, pUC19 or pBR322.

39. A vector of Claim 34, where the vector is a suitable shuttle vector.

40. A shuttle vector of claim 39, selected from pMK4, or pYL112Δ119.

41. An expression vector of Claim 34 where the expression vector is a plasmid with a strong promoter.

42. An expression vector of Claim 41 where the plasmid with the strong promoter is selected from p*Trc*99A, pDR540, or pET-21(+).

43. A plasmid of claim 35, named pRODCAP18, comprising a cloning plasmid containing a DNA sequence substantially the same as the DNA sequence of SEQ. ID. NO. 1, placed into the cloning plasmid, pUC18.

44. A plasmid of claim 39, named pMKRODC, comprising a shuttle plasmid containing a DNA sequence substantially the same as the DNA sequence of SEQ. ID. NO. 1, placed into the shuttle vector pMK4.

45. A plasmid of claim 35, where the plasmid is produced as the product from the process of excising the nucleic acids comprising the nucleic acids from SEQ. ID. NO. 1, that code for the protein described in SEQ. ID. NO. 2 from a pRODCAP18 plasmid, of claim 43.

46. A plasmid of claim 41, named pBSRODC1 or pBSRODC1, where the plasmid is produced as the product from the process of excising the nucl ic acids comprising the nucleic acids from SEQ. ID. NO. 1, that code for the protein described in SEQ. ID. NO. 2 from a pRODCAP18 plasmid, of claim 43, which is then placed into an expression vector with a strong promoter.

47. A plasmid of claim 46 where the expression vector with a strong promoter is p*Trc*99A.

48. A plasmid of claim 41, where the plasmid is produced as the product from the process of excising the nucleic acids comprising the nucleic acids from SEQ. ID. NO. 1, that code for the protein described in SEQ. ID. NO. 2 from a pMKRODC plasmid of claim 44, which is then placed into an expression vector with a strong promoter.

49. A plasmid of claim 48 where the expression vector with a strong promoter is p*Trc*99A.

50. A diagnostic kit utilizing the TAP enzyme and CDPglycerol to detect and monitor disease caused by gram positive bacteria.

(CELL)

51. A bacterial cell transformed with a vector of claim 34.

52. A bacterial cell of claim 501where the bacterial cell is an *E. coli* cell.

53. A bacterial cell of claim 52, where the *E. coli* cell is type DH10B.

54. Any of the DNA of claims 22-23, incorporated into a vector of claims 34-49.

55. A bacterial cell of claim 51, transformed with any of the plasmids from claims 34-49.

56. A bacterial cell of claim 55 that is an *E.coli* cell

57. A bacterial *E.coli* cell of claim 56 that is type DH10B.

58. The proteins disclosed in the Southern Blot shown in Figure 2.

59. The method of claims 7-14, where the lipoteichoic acid is treated with a suitable agent to remove alanine before it is combined with the other ingredients.

5

60. The inventions and discoveries disclosed in this application.

Figure 1

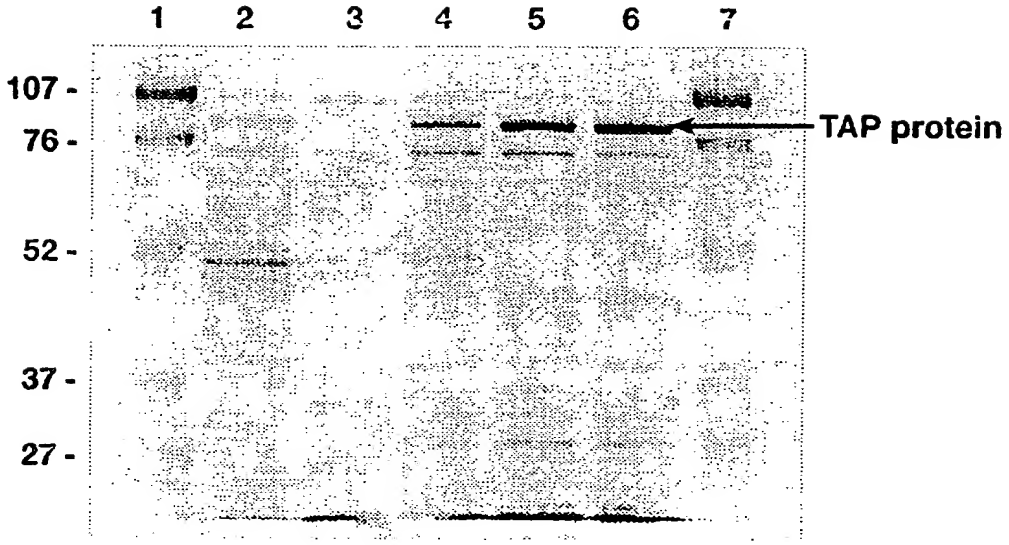
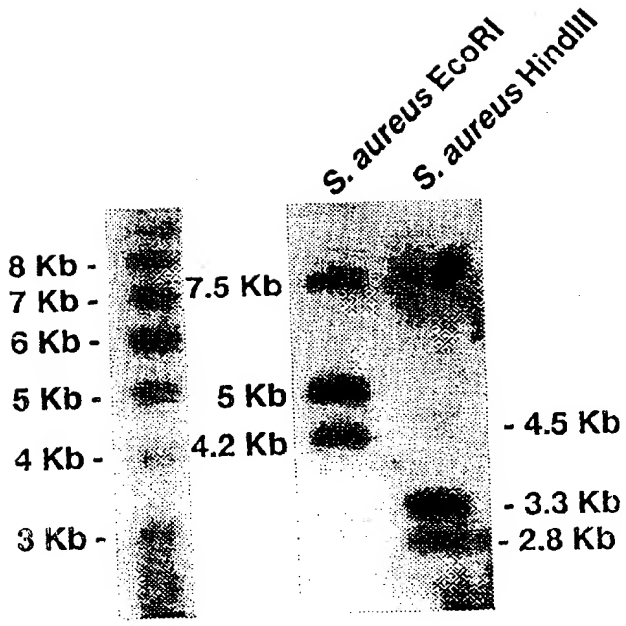


Figure 2



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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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16 April 1998 (16.04.98)

(54) Title: TEICHOIC ACID ENZYMES AND ASSAYS

(57) Abstract

This invention discloses a novel substrate and assay for the TAP enzyme. In addition novel DNA, proteins and peptides from genes and proteins associated with bacterial teichoic acid biosynthetic pathways, specifically the *rodC* gene and proteins and variations thereof are disclosed.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/07123

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/48 C12N9/12 C12N15/54 C12N15/63 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAUCK, JOHN ET AL: "Acceptor-dependent polyglycerolphosphate polymerase" PROC. NAT. ACAD. SCI. U. S. A. (1972), 69(9), 2386-90 CODEN: PNASA6, 1972, XP002040448 See passage from first full paragraph of left column on page 2390 until the end. see the whole document --- -/--	1-9, 12-21,58

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1998	04. 03. 98

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/07123

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HONEYMAN, A.L. AND STEWART G.C. : "The nucleotide sequence of the rodC operon of Bacillus subtilis" MOL. MICROBIOL., vol. 3, 1989, pages 1257-1268, XP002053854 Nucleic acid residues 2178 until 4415. see figure 3 & DATABASE SWISS-PROT (EMBL) EMPRO X15200; 940098, 3 May 1989 HONEYMAN A.C. AND STEWART, G.C.: "The nucleotide sequence of the rodC operon of Bacillus subtilis" see abstract	15-59
A	POLLACK, JORDAN H. ET AL: "Changes in wall teichoic acid during the rod-sphere transition of Bacillus subtilis 168" J. BACTERIOL. (1994), 176(23), 7252-9 CODEN: JOBAAY;ISSN: 0021-9193, 1994, XP002040449 see the whole document	1-59
A	POOLEY, HAROLD M. ET AL: "CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase, which is involved in the synthesis of the major wall teichoic acid in Bacillus subtilis 168, is encoded by tagF (rodC)" J. BACTERIOL. (1992), 174(2), 646-9 CODEN: JOBAAY;ISSN: 0021-9193, 1992, XP002040450 see page 646, left-hand column	1-59

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/US 97/07123

Box I Observations where certain claims wer found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1-14, 59, 60 (part)
2. Claims 15-58, 60 (part)

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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